ELECTRON MICROSCOPIC ANALYSIS OF THE PRODUCTS OF DNA SYNTHESIS BY DNA POLYMERASES FROM CALF THYMUS AND HERPES SIMPLEX VIRUS TYPE I

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I. Abstract

Replication of single stranded M13 DNA by the calf thymus DNA polymerase α:primase complex and herpes simplex virus type 1 (HSV-1) DNA polymerase was examined by electron microscopy. The replicative intermediates isolated from the reaction were spread for electron microscopy in the presence of E. coli single stranded binding (SSB) protein and ethidium bromide. The electron microscopic analysis of the replicative intermediates from the calf thymus DNA primase primed M13 DNA replication showed an average of 2.5 primers per M13 DNA circle. The measurement of the double stranded length from individual replicative intermediates by electron microscopy was within the accuracy of 10% standard deviation. The product length distribution obtained from the HSV-1 DNA polymerase catalyzed replication of M13 DNA primed with a specific pentadecamer and in the presence of E. coli SSB protein showed a near Poisson distribution. Replication of the same primer-template system or DNA primase primed M13 DNA template by calf thymus DNA polymerase α showed a broad distribution of product lengths. Analysis of replicative intermediates after digestion with restriction endonuclease Hae III showed that replication of singly primed M13 by calf thymus DNA polymerase α is affected by a major hairpin structure located at the origin of replication. Elongation of a primer at an aberrant priming site located upstream from the primary priming site was observed during

replication. HSV-1 DNA polymerase replication in the presence of E. coli SSB appeared not to be affected by this hairpin structure as analyzed by Hae III Restriction digestion. Electron microscopic analysis of early replication products synthesized by HSV-1 DNA polymerase and calf thymus DNA polymerase α:primase complex showed that the majority of products were partially replicated. Product analysis of reactions with DNA template concentrations increased 2-3 fold also showed partially replicated structures with no full length products. These results support the hypothesis that both DNA polymerases replicate DNA in a distributive manner.

ELECTRON MICROSCOPIC ANALYSIS OF THE PRODUCTS OF DNA SYNTHESIS BY DNA POLYMERASES FROM CALF THYMUS AND HERPES SIMPLEX VIRUS TYPE 1

By

Samantha Julie Li

Thesis submitted to the Faculty of the Department
of Biochemistry Graduate Program of the
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[1]The abbreviations used for nucleotides and polynucleotides are those of IUPAC-IUB Commission of Biochemical Nomenclature (CBN) 1970, J. Biol. Chem. 245:5171-5176. Other abbreviations used are: RF, replicative form; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; NP40, nonidet P-40; BSA, bovine serum albumin; β-ME, β mercaptoethanol; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; KPi, potassium phosphate; DTT, dithiotheritol; TCA/PPi, trichloroacetic acid/pyrophosphate; kDa, kilodalton; NEM, N-ethylmaleimide; RI, replicative intermediates; Tris-Cl, tris(hydroxymethyl) aminomethane-chloride; NH_4OAc, ammonium acetate; PAA, phosphonoacetic acid.

II. Introduction

Most of the advances in the understanding of the mechanism of DNA replication, the proteins involved, their structures and functions, have come from studies on prokaryotic DNA replication. This has been possible through the use of conditional lethal mutants, to implicate various proteins in the process, and the development of efficient in vitro DNA replication systems. Many of the systems have employed bacteriophage DNA replication, because bacteriophage DNA replication are somewhat simpler processes than that of E. coli. More recently, genetic engineering techniques have been used to clone and overexpress the genes for several of the genes involved in DNA replication of several bacteriophage, as well as E. coli and this has enabled more detailed study of many of these proteins to begin.

It has not been possible to study eukaryotic DNA replication in the same detail. The lack of conditional lethal mutants in higher eukaryotes has hindered direct implication of many proteins thought to be involved in the DNA replication process. Attempts have been made to develop eukaryotic in vitro DNA replication systems, but the information obtained from such systems has been limited. Many of the systems have used the replication of the DNA of virus SV40, which employs mainly host enzymes. The replication of Herpes Simplex Virus (HSV) DNA has been proposed to be a useful model system for the replication of eukaryotic DNA. This virus encodes a minimal of seven proteins essential for the replication of it's DNA. These include a DNA polymerase, a single stranded DNA binding

protein, a DNA helicase, a replication origin binding protein and three other proteins whose functions are as yet unknown.

Key elements in the understanding of the DNA replication process are the implication of a specific DNA polymerase as the replicative enzyme, the characterization of it's properties, especially enzyme mechanism, and the isolation and characterization of proteins with which the DNA polymerase may interact and which may alter the properties of the DNA polymerase.

II.A. DNA polymerases in higher eukaryotes

The first eukaryotic DNA polymerase to be isolated and characterized was the enzyme now known as DNA polymerase α (Bollum, 1960). Since then, several distinct types of DNA polymerase have been discovered. Presently there are three well-studied and ubiquitous classes of DNA polymerases in higher eukaryotes: α, β and γ (Holmes et al., 1983). These DNA polymerases can be distinguished from each other by their chromatographic properties, molecular weight, inhibition by NEM and salt as well as their ability to copy various templates (Fry, 1986). The functional role of each polymerase in the cells is quite different (Table I). Both DNA polymerase α and γ are involved in the replication of DNA; DNA polymerase α is the main replicative enzyme in the nucleus (Chang and Bollum, 1973) while DNA polymerase γ is involved in DNA replication in the mitochondria (Bolden et al., 1977). DNA

Table I. Properties of animal DNA polymerases α, β and γ.

Properties	α	β	γ
Molecular Weight (kDa)	140-185	45	>140
S Values	6-9	3.5	7-9
Isoelectric Point	5.6-6	9-9.4	5.4-6.1
% of total activity:			
1.) growing cells	>85	10-15	2-5
2.) resting cells	0-5	90-95	0-10
High Ionic Strength	Inhibition	Stimulation	Stimulation
Effect of NEM	Inhibiton	No effect	Inhibition
Effect of Aphidicolin	Inhibition	No effect	No effect
Effect of Butilanilinouracil	Inhibition	No effect	No effect
Template-primer:			
1.) Activated native double stranded gapped DNA.	yes	yes	yes
2.) deoxyhomopolymer: riboprimer.	yes	no	yes
3.) ribo-homopolymer: deoxyprimer.	no	yes	yes

Adapted from Falaschi and Spadari, 1978.

polymerase β is found primarily in the nucleus and is thought to be involved in repair type synthesis (Siedlecki et al., 1980). Although much is understood about the functional role of the cellular DNA polymerases in DNA replication, many interesting questions concerning how a DNA chain is extended, that is the mechanism of DNA replication, remain unanswered.

Some viruses upon infection of animal cells have been found to induce a new DNA polymerase that is different from the host DNA polymerase (Citarella et al., 1972). This new DNA polymerase has been found in cells infected with different types of viruses such as those from vaccinia, Herpes simplex virus type 1 and 2, and others (Citarella et al., 1972; Hay and Subak-Sharpe, 1976). By using a temperature sensitive mutant of HSV-1, Aron et al. showed that the inducible DNA polymerase is encoded by the virus and that it is necessary for viral DNA replication (Aron et al., 1975). The general properties of DNA polymerase α and HSV-1 DNA polymerase will be reviewed in the following paragraphs. They are summarized in Table II.

II.A.1. DNA Polymerase α

DNA polymerase α has been isolated from many higher eukaryotes (Fry, 1986). Problems encountered in purification and heterogeneity in size have been attributed to proteolysis and to association of the DNA polymerase with other proteins, which may

Table II. Comparison of herpes simplex virus type 1 (HSV-1) DNA polymerase with DNA polymerase α.

	HSV-1 DNA Polymerase	DNA Polymerase α
Molecular Weight (KDa)	130-140	140-185
Number of subunits	1	1
3'->5' Exonuclease	Yes	No
Inhibition by PAA	Yes	No
Effect of 0.2 M NaCl	Activates	Inhibits
Aphidicolin Sensitivity	Yes	Yes
Use of RNA primer	Yes	Yes

See text for references

be involved in DNA replication (Holmes et al, 1974; Fisher and Korn, 1977; Brakel and Blumenthal, 1977; Fichot et al., 1979). DNA polymerase α has a molecular weight of 140,000 to 185,000 daltons and can be co-purified with a DNA primase, an enzyme capable of synthesizing short oligoribonucleotide primers (Conaway and Lehman, 1982).

DNA polymerase α is the main replicative enzyme (Fry, 1986). Using immunoaffinity chromatography to purify the calf thymus DNA polymerase α:primase complex, Chang et al. (1984) isolated five major polypeptides; 185 kDa, 160 kDa, 68 kDa, 55 kDa and 48 kDa. When antibodies were made against these polypeptides, Holmes et al. (1986) showed by using immunoblot analysis the 160 kDa polypeptide to be derived from the 185 kDa polypeptide and the 48 kDa polypeptide to be derived from the 68 kDa polypeptide. Antibodies to the three smaller polypeptides inhibit DNA primase activity. From these results, Holmes et al. proposed that the native calf thymus DNA polymerase α:primase complex contains three polypeptides: 185 kDa, 68 kDa and 55 kDa. In addition, the DNA polymerase activity resides in the 185 kDa and the two smaller polypeptides constitute the primase activity. Results of antibody inhibition studies and UV crosslinking of DNA primase to α^{32}P-GTP, showed that the calf thymus primase activity is associated with the 48 kDa polypeptide (Nasheuer and Grosse, 1988). On the other hand, Hirose et al. (1988) found the primase activity was associated

exclusively with an immunopurified 60 kDa polypeptide from chicken while the 160-180 kDa polypeptides contained the DNA polymerase activity. It is clear from these studies that although it is agreed that more than one polypeptide is found in the DNA primase preparation, it has not yet been concluded which polypeptide(s) are responsible for DNA primase activity.

II.A.2. Herpes Simplex Virus type 1 DNA Polymerase

The induction of a novel DNA polymerase by HSV was first recognized by Keir and Gold (Keir and Gold., 1963) and this enzyme was later shown to be immunologically different from the host cellular enzyme (Keir et al., 1966). The purified enzyme has been found to be a single polypeptide with a molecular weight of about 136,000 to 140,000 dalton (Ostrander and Cheng, 1980). The gene for the HSV DNA polymerase has been cloned and the sequence determined and the predicted molecular weight agrees with that of the purified enzyme (Gibbs et al., 1985). The HSV DNA polymerase resembles DNA polymerase α in its sensitivity to the drug aphidicolin (Pedrali-Noy and Spadari, 1979; Hay and Subak-Sharpe, 1976). However, the HSV DNA polymerase can be easily distinguished from the DNA polymerase α by several properties. The first is the requirement for high salt concentration (~0.2 M NaCl) by HSV-1 polymerase (Powell and Purifoy, 1977), which gives the viral enzyme maximal activity, but inhibits the host enzyme. The second

is the sensitivity of HSV-1 DNA polymerase to phosphonoacetate, which inhibits by interacting with the PPi binding site of the enzyme (Mao et al., 1975). Lastly, the HSV DNA polymerase possesses a 3'-5' exonuclease activity (Derse and Cheng, 1981). Although the HSV-1 DNA polymerase can be distinguished from mammalian DNA polymerase α, the two DNA polymerases share a common property, both can use a short piece of RNA as a primer for DNA synthesis (Weissbach et al., 1973).

II.B. The mechanism of enzymatic synthesis of DNA

The mechanism of DNA synthesis by DNA polymerase is a cyclic process. The process involves an addition of deoxynucleoside monophosphate (dNMP) to the growing end of the initatior chain via a nucleophilic attack by the 3' hydroxyl group of the primer terminus on the α phosphoryl group of an incoming dNTP, with the elimination of the terminal pyrophosphate. The result is a new primer terminus with a free 3' hydroxyl group which is ready to initiate another attack on an incoming dNTP. However, before the primer terminus can accept another dNTP, the DNA polymerase must regain it's initial configuration with respect to the primer-template (Kornberg, 1980). This brings up the question of how the polymerase achieves it's initial configuration. At present, there are two views of the action of the DNA polymerase (Figure 1):

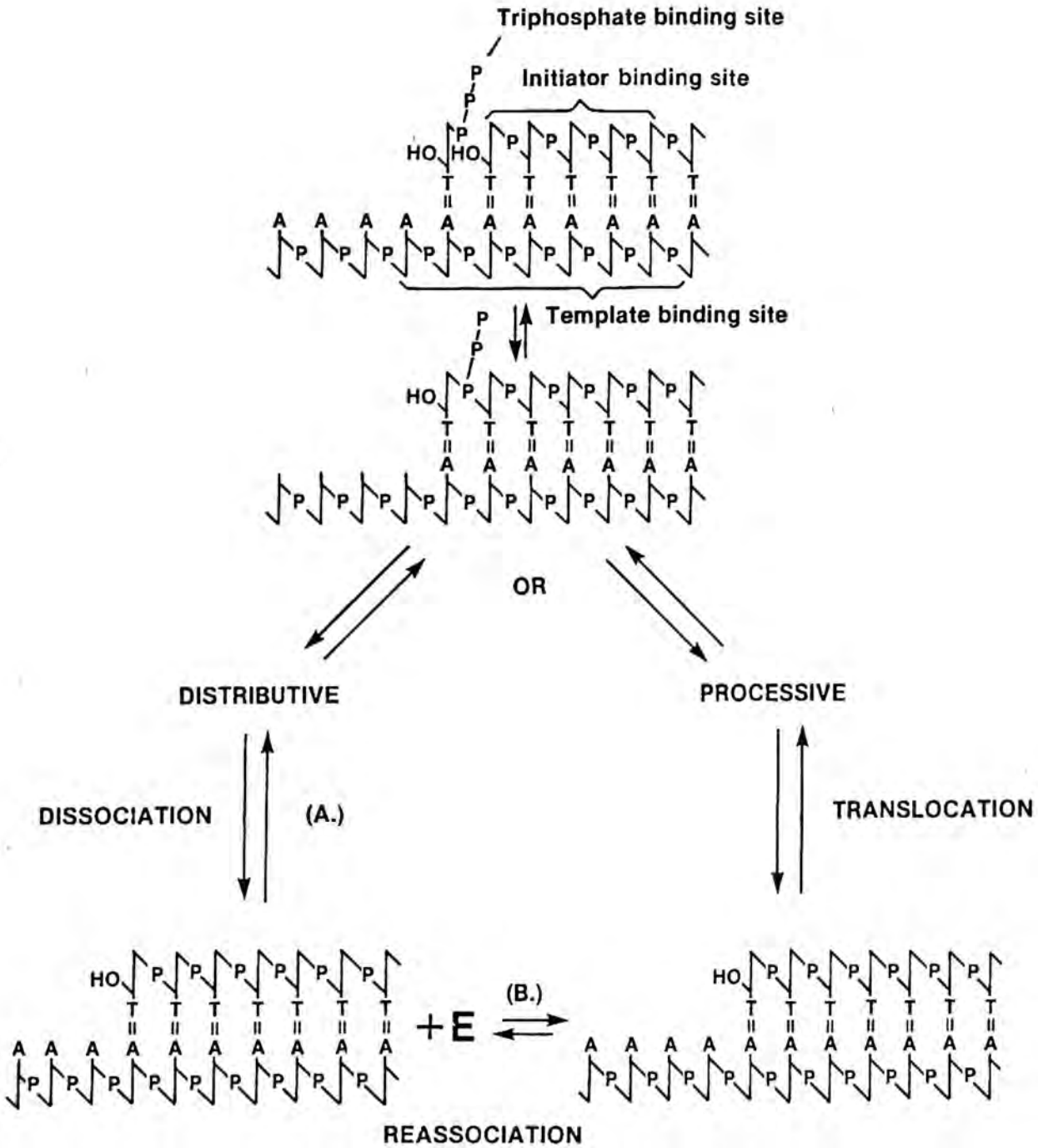


Figure 1

Schematic representation of DNA synthesis by DNA polymerase. Adapted from Chang, 1976.

1.) After adding a given deoxynucleoside monophosphate (dNMP), the growing end of the template remains bound to the active site of the enzyme, and then the enzyme is translocated on the primer-template. The mechanism may involve a secondary binding site on the enzyme which allows the template to remain at the active site while the enzyme changes configuration. Consequently, the enzyme is able to add another dNMP and continue to replicate without dissociating until the template is copied. This is the so called "Processive Mechanism".

2.) The alternative view is that the enzyme dissociates from the template and reassociates at either the previous or new primer-template. This mechanism is called the "Distributive or Non-processive Mechanism".

Several experimental techniques have been used to study the polymerization cycle: template challenge, cycling time perturbation and product size. The logic behind template challenge is to examine whether the DNA polymerase remains associated with the template that it is replicating until that template is completely replicated or begins replicating a new template before compieting the first one. The experiment is carried out in an excess of primer-template molecules to the number of DNA polymerase molecules. Using this method, Chang (1976) demonstrated that both DNA polymerases α and β acted distributively. The conclusion that DNA polymerase α was a distributive enzyme was supported later by McKune and

Holmes (1979) using similar techniques. This type of experiment has also been used to show that E. coli DNA Polymerase I is non-processive (McClure and Jovin, 1975). Using similar methodology, it was found that HSV-1 DNA polymerase completely replicated the original template when challenged with a competing template, thus suggesting that the HSV-1 DNA polymerase is processive (O'Donnell et al., 1987). However, this technique poses problems (Bambara et al., 1978) because one has to be certain that all the enzyme molecules are active at any given time. For example, if the DNA polymerase has two forms, an active and inactive one, then it is possible that the DNA polymerase can be converted to either form during any time of the replication. Therefore, any DNA polymerase which became active after the addition of the competing template is able to replicate the competing template. A second problem is that it is possible that enzyme dissociation is enhanced by the challenging template.

The second technique is called the cycling time perturbation, or limited reaction rate, which involves a comparison of the polymerization rate catalyzed by the DNA polymerase on a defined template in the absence of one or more dNTP vs. the rate in the presence of all four dNTPs. This method proposes that the reaction rates obtained in the absence of various combinations of deoxynucleoside triphosphates should depend directly on the average number of nucleotides added to a primer terminus before the growing point stops from the lack of a missing deoxynucleoside

triphosphate. Therefore, the goal is to express the average number of nucleotides polymerized with a full complement of dNTPs, in terms of polymerization rate with the limited versus complete complement of dNTPs. Bambara et al (1978), who developed this technique, found DNA polymerase β from KB cells to be non-processive. The same method was again used by Hockensmith and Bambara (1981) to compare the processivity of two forms of calf thymus DNA polymerase α. It was found that both forms were distributive, adding 11 and 17 nucleotide respectively before dissociating. As noted by Bambara, this method has its limitations in that it cannot be used with a homopolymer DNA substrate. In addition, cross-contamination of one dNTP with another is possible, although this problem can be eliminated with extensive purification of dNTPs.

The last method involves the analysis of reaction products. The experimental rationale again requires the DNA synthesis reaction to be carried out with an excess of primer template molecules to the number of DNA polymerase molecules. Therefore, in a processive synthesis, if the enzyme does not dissociate from an elongating primer, then the number of primers which are elongated is maximally equal to the number of enzyme molecules present (if all molecules are active). On the other hand, in a distributive mode of synthesis, where the enzyme dissociates after each nucleotide addition, one would expect a Poisson distribution of product length.

For example, if 10% replication of total template DNA has occurred, then all the primers should be elongated with an average of 10% of their total single stranded length. These two possibilities can be distinguished using gel electrophoresis as a means to determine the product length distribution. Subjecting reaction products to an alkaline agarose gel electrophoresis, Villani and colleagues (1981) found that DNA polymerase α from Drosophila melanogaster was distributive and gave a heterogeneous distribution of products on a multiprimed M13 template. Using similar methodology, calf thymus DNA polymerase α was also shown to be a distributive enzyme incorporating approximately 19 nucleotides per binding of DNA polymerase to primer-template (Hohn and Grosse, 1987). Furthermore, using neutral agarose gel electrophoresis to examine replication products, the claim that HSV-1 DNA polymerase is a highly processive enzyme was supported because full length double stranded DNA was formed while most of the template remained unchanged as single stranded DNA (O'Donnell et al., 1987). This technique, although simple to perform, requires good recovery and resolution of individual replicative products.

II.C. The Use of Single Stranded DNA Binding Proteins in the Staining of Single Stranded Polynucleotide for Electron Microscopy

Bacteriophage T4 coded gene 32 protein was first purified

using DNA cellulose chromatography (Alberts et al., 1968). Gene 32 protein was shown to bind preferentially and more tightly to single stranded DNA than to double stranded DNA. The binding by Gene 32 protein was cooperative based on sedimentation. In addition, using sedimentation analysis, Alberts and Frey (1970) showed that one molecule of gene 32 protein monomer binds to approximately 7 nucleotides of single stranded DNA, and it was calculated that gene 32 protein has at least 80 fold greater affinity for a site adjacent to an already bound protein molecule than free DNA. Visualization of gene 32 protein-single stranded DNA complexes by electron microscopy supported the experimental result that gene 32 protein binds cooperatively to single stranded DNA. With protein concentration in excess, the single stranded DNA is coated uniformly with the protein to form a flexible rod-like complex (Delius et al., 1972). The same studies also showed that gene 32 protein can invade A-T rich regions of duplex DNA but not native supercoiled double stranded DNA. These properties led to the use of such single stranded binding proteins in the electron microscopic visualization of single stranded DNA.

Under standard spreading methods, the single strand may be deformed due to the spreading technique or it may be too difficult to discriminate the single from the double strand based on thickness alone. Wu and Davidson (1975) used gene 32 protein to develop a method for mapping the position of 16S, 23S and 5S rRNA and several tRNA genes present on the DNA from transducing phage

80d3idvsu+7. The concept being that a position on the DNA could be mapped by first hybridizing the RNA to its DNA and then observing the position of the duplex region along the single stranded DNA by electron microscopy. This gene 32 protein staining method was used to map single stranded regions in Bacteriophage PM2 (Brack et al., 1975) which is the most tightly supercoiled DNA known. The result showed that not only were there one to three small single stranded regions present but they were mapped to a unique cleavage site on the DNA for the restriction endonuclease R. Hap II. This technique also has been applied to map the relative position of several histones genes and intervening spacer sequences on the DNA from Drosophila (Wu et al., 1976). In addition, the method was used to delineate these structures of the single stranded ends of Adenovirus type 2 DNA (Wu et al., 1977).

Another protein sharing many of the characteristics of gene 32 protein is E. coli Single Stranded Binding Protein (SSB). Sedimentation studies showed that SSB binds preferentially and cooperatively to single stranded DNA (Segal et al., 1972). However, unlike gene 32 protein which binds to DNA as a monomer, SSB binds to DNA as a tetramer (Weiner et al., 1975). Electron microscopy studies also supported the view that SSB binds to DNA as a tetramer, with one molecule of SSB monomer binding to 8 nucleotides of DNA (Segal et al., 1972). Another difference between T4 gene 32 protein and E. coli SSB protein is that the size of the single stranded DNA binding site of SSB and the cooperative nature

of protein:protein interaction are affected by salt concentration but salt has no effect on the the size of the binding site of gene 32 protein (Lohman et al., 1988). Like gene 32 protein, SSB has been used to stabilize and to differentiate single stranded DNA from double stranded DNA. For example, this method has allowed the visualization of single stranded DNA localized in both terminal and internal regions of the duplex, which were generated by Rec BC enzyme (Rosamond et al., 1979; Taylor and Smith, 1980). Lastly, SSB was also used to visualized single stranded ends of reconstituted histone-DNA complexes (Dunn and Griffith, 1980).

The electron microscopic techniques used in all these studies have demonstrated that DNA binding proteins can provide a sensitive test to distinguish single stranded DNA from double stranded DNA. In addition, these proteins have been shown to be potential cytological reagents for studies of nucleic acids by electron microscopy.

II.D. Research Goal

As previously mentioned, the template challenge method relies on observing a perturbation in the polymerization on the template upon introduction of a challenging template. However, before any conclusion can be made, the relative affinity of the enzyme for both templates must be determined beforehand. The cyling time perturbation method devised by Bambara et al. relies on a steady state measurement to deduce the average time the enzyme

spent on the template of interest. However, this method is open to the criticism that the enzyme may be present in additional complexes such as binding nonspecifically to double stranded DNA or at 3'-OH end, or may just be free in solution. These two methods clearly require important kinetic information about the perturbing template. Therefore, necessary characterization of the perturbing molecule's interaction with the enzyme is required before processivity can be measured. On the other hand, product analysis is a more direct method and does not involve any perturbing molecules. The only requirement for the application of product analysis is the resolution of product length. The procedures using gel electrophoresis or filtration are often limited by either low resolving power or ability to clearly separate oligonucleotides, respectively. Although oligonucleotides can be separated by fractionation on DEAE cellulose, the amount of each oligonucleotide is difficult to determine due to poor resolution. Thus, it is the intent of this study to develop an alternative method for product analysis that is quantitative and versatile enough to allow variation of reaction conditions, such as temperature and substrate concentration, but eliminates the problem of resolution seen in gel electrophoresis and gel filtration. The technique consists of electron microscopic analysis of the products, and allows visualization of the reaction products and direct measurement of the length of replicated double strand. In addition, instead of densitometer tracing or gel slicing of radioactive labeled elongated

products, the relative number of products varying in length can simply be summed and integrated into a product distribution profile.

Using this method, the second goal is then to analyze the question of the processivity of two DNA polymerases: calf thymus DNA polymerase α and HSV-1 DNA polymerase. DNA polymerase α has been found to be a distributive enzyme using methods previously described. On the other hand, DNA HSV-1 polymerase has been suggested to be a processive enzyme by product analysis using polyacrylamide gel electrophoresis and by template challenge. In view of these results, it was of interest to apply electron microscopic techniques to examine the question of processivity in both DNA polymerase α and HSV-1 DNA polymerase.

III. Experimental Procedures

III.A. Materials

Unlabeled rNTPs, dNTPs, Cytochrome C, DMSO, PMSF, and uranyl acetate were obtained from Sigma. M13 single stranded mp9 DNA, M13 primer, d(TCCCAGTCACGACGT), and M13 RF mp9 DNA were from Boehringer Mannheim Biochemical. [^{3}H]-dATP and [α^{32}P]-dCTP were from New England Nuclear. Geneclean kit was obtained from Biol 101, Incorporated. Restriction endonuclease Hae III was obtained from Bethesda Research Laboratory. Herpes simplex virus type 1 DNA polymerase and E. coli SSB were provided by Steven Wietstock of the Department of Biochemistry, Uniformed Services University of the Health Sciences. Protein A-Sepharose and Sephadex G-50 were from Pharmacia. Agfa-Gevart print paper (rapitone type PF1-4) and Kodak EM film were from Fuller D'Albert. Copper grids (200 mesh) were from Balzer. Map measure (swivel handle) was from K + E. Magnifying lens (scale lupe 10X) was from Peak. Parlodion 3%-amy acetate was from Ernest F. Fuller Incorporated. Pt/Pd (80% Pt/20% Pd) was from Pelco. Tungsten V filament was from Ted Pella Incorporated. Scintillation fluid was from Beckman. All other chemicals were reagent grade.

III.B. Methods

III.B.1. Purification of the calf thymus DNA polymerase α:primase complex.

Calf thymus DNA polymerase α:primase complex was purified in an immunoaffinity procedure as described previously (Chang et al., 1984). Briefly, the following procedures were carried out at 4°C. 500 grams of frozen Calf Thymus glands were homogenized in a buffer (Buffer A) containing 40 mM NaCl, 40 mM KPi at pH 7.4, 1 mM PMSF and 1% DMSO. The extract was then centrifuged at 575 x g at 4°C for 15 minutes after which the supernatant was passed through 4 layers of cheese cloth to remove undisrupted materials, connective tissue and lipid particles. After adding protamine sulfate (to a final concentration of 0.125%) to the supernatant for the removal of nucleic acid, the suspension was centrifuged at 2,050 x g for 30 minutes at 4°C. The supernatant, which was slightly cloudy, was clarified by the addition of NP40 to a final concentration of 0.1%. This protamine sulfate solution was loaded onto a 5 ml mouse monoclonal antibody column against calf thymus DNA polymerase α (Mab #42). After the extract was loaded onto the column, it was washed with Buffer A. Free DNA primase was eluted with 1 M NaCl in 0.05 M Tris-Cl buffer at pH 8.0. The column was then washed with 3 M NaCl in 0.05 M Tris-Cl buffer at pH 8.0. Protein remaining on the column was eluted with 3.2 M $MgCl_2$ in a buffer containing 50 mM Tris-Cl at pH 8.0. Fractions containing the proteins were determined by absorbance reading at

280 nm and pooled. The pooled fractions were loaded onto a 2.3 x 60 cm Sephadex G-50 column to remove the $MgCl_2$. The protein fractions were collected and passed through a 5 ml Protein A Sepharose column to remove endogeneous IgG present in the thymus gland. Protein was then concentrated by precipitation at 80% saturation with $(NH_4)_2SO_4$. The pellet was resuspended in 20 mM Tris-Cl at pH 7.5, 50 µg/ml BSA, 15% glycerol and 2 mM β-ME and then dialyzed against a buffer containing 20 mM Tris-Cl (pH 7.5), 50 µg/ml BSA, 50% glycerol and 2 mM β-ME and stored at -20°C.

III.B.2. Measurement of DNA synthesis.

In the primase primed (multiprimed) M13 DNA reaction, replication of M13 DNA was measured in 300 µl reaction mixtures containing 50 mM Tris-Cl, pH 8.0, 1 mM DTT, 8 mM MgCl, 0.1 mM each of dGTP, dCTP, dTTP, rGTP, rCTP, rUTP and ^{3}[H]-dATP (40 cpm/pmol), 1.0 mM ATP, 100 µg/ml BSA and 4.76 nM of single stranded circular M13 mp9 DNA. The reaction was initiated by adding 20 µl of calf thymus DNA polymerase α:primase complex containing a total of 118 units of polymerase. One unit of polymerase activity is defined as the incorporation of 1 nmol of deoxythymidine monophosphate into activated DNA per hour at 37°C under condition described previously (Chang et al., 1984). The reaction was incubated at 37°C and 30 µl aliquots were removed at the times indicated in the appropriate figure legend and pipetted onto glass fiber filters. The radioactivity

in the nucleic acid was then precipitated onto the filters by placing in an ice cold solution of 5% TCA/0.1% PPi for 10 minutes. The filters were washed twice with 1 N HCl on ice for 10 minutes followed by two rinses in 95% ethanol for 5 minutes. Filters were dried and added to 5 ml of Beckman Ready Organic Scintillation fluid and then counted on a Packard Tri-Carb Liquid Scintillation Spectrophotometer. The data were represented as percent replication, which was determined by pmol of dNMP incorporated divided by the total pmol of single stranded M13 DNA present.

In the singly primed M13 DNA reaction, the primer-template was prepared as followed: 13 nM pentadecamer 5'd(TCCCAGTCACGACGT) and 2.86 nM of single stranded circular M13 mp9 DNA were annealed at 65°C for 15 minutes in 150 mM KCl. The annealing reaction was then cooled for 30 minutes at room temperature. Similar to the multiprimed M13 DNA reaction, DNA synthesis by the DNA polymerase α:primase complex was measured in a 250 µl volume containing the same concentration of reaction buffer, except for the absence of four rNTPs. The reaction was started by the addition of the DNA polymerase α:primase complex (a total of 118 units). The reaction was carried out at 37°C and 30 µl aliquots were removed at various times and processed as mentioned above.

For the HSV-1 DNA polymerase reaction, the measurement for DNA synthesis was carried out in a 200 µl volume. The primer-template was added to the same reaction mixture as

described for the DNA polymerase α:primase complex singly primed reaction. KCl and E. coli SSB were added to a final 70 mM and 115 μg/ml respectively, to give maximal activity of the HSV-1 DNA polymerase. The reaction mixture was preincubated at 35°C for 15 minutes to allow SSB to coat the M13 DNA. The reaction began with the addition of 40 μl of HSV-1 DNA polymerase (a total of 4.4 units). The reaction was incubated at 35°C and 20 μl aliquots were removed at various times and processed as above.

III.B.3. The effect of SSB in the replication of M13 DNA by HSV-1 DNA polymerase.

M13 mp9 single stranded circular DNA (2.86 nM) and 13 nM of M13 mp9 pentadecamer were annealed in 150 mM KCl by heating to 65°C for 15 minutes and allowing the solution to cool for 30 minutes. The DNA-primer solution was added to a reaction mix as described for the HSV-1 DNA polymerase assay, with the exception that the mix contained 104 μg/ml of SSB. The reaction was incubated at 35°C for 15 minutes before initiation with a total of 4.4 units of HSV-1 DNA polymerase. The reaction was incubated at 35°C and 20 μl aliquots were removed at various times and processed as described above.

III.B.4. Reaction Product Isolation

Samples were prepared as described for the replication

assays, except the total reaction volumes. 200 µl aliquots were removed at defined times. The reactions were stopped with the addition of 25 mM EDTA (final concentration) and chilled on ice. The purification of reaction product follows the procedure described by the Geneclean Kit (Biol 101, Inc) derived from Vogelstein and Gillespi (1979). In summary, two and a half volumes of saturated NaI solution were added and the mixtures were vortexed well. A 10 µl aliquot of glassmilk solution, containing a suspension of silica matrix in water, was added to each sample and the mixtures were incubated for 5 minutes on ice. In the presence of the chaiotropic salt, NaI, silica beads bind well to DNA molecules. The glassmilk/DNA suspensions were centrifuged for 1 minute and the supernatants removed. In case all the DNA had not been bound to the silica matrix, the binding procedure was repeated with the addition of 10 µl of glassmilk to the supernatant. The pellets were washed 3 times with 600 µl of ice-cold NEW (NaCl/Ethanol/Water) solution, consisting of 20 mM Tris-Cl at pH 7.8, 0.2 M NaCl, 2 mM EDTA and 50% ethanol. The wash procedure involved resuspending the pellet in the NEW solution and discarding the supernatant after centrifugation for 10 seconds. DNA was then eluted from the glassmilk by resuspending the pellet in 50 µl of 10 mM Tris-Cl at pH 8.0 and 1 mM EDTA (TE buffer). The samples were then incubated at 50°C for 3 minutes, followed by a 1 minute centrifugation. The supernatants containing the DNA were removed and placed in new tube. A second elution was done as described above, with the addition of 50 µl TE

buffer to the glassmilk pellet. The reaction products in TE buffer were pooled. A typical recovery of DNA was 75%, as determined by the difference in acid precipitable radioactivity before and after the geneclean procedure.

III.B.5. Neutral Agarose Gel Electrophoresis.

Neutral agarose gels were prepared as horizontal slabs (15 x 20 x 0.5 cm) from a solution of 1% Agarose in 50 mM Tris-borate at pH 8.6 and 1 mM EDTA (Buffer B). The loading buffer contained 0.5% Xylene Cyanole FF, 0.5% Bromophenol Blue and 20% Ficoll. Aliquots removed from the DNA replication reaction at various time were terminated by the addition of EDTA at a final concentration of 25 mM and chilled on ice. SDS (to a final concentration of 1%) was added to the samples, then the samples were heated at 37^{o}C for 10 minutes. 2 µl of 10X loading buffer was then added to 20 µl samples. The gel was electrophoresed in buffer B at a constant voltage of 30 Volts for 18 hours. DNA markers used were single stranded M13 mp9 DNA and the replicative form of M13 DNA. The gel was stained with ethidium bromide (1 µg/ml in buffer B) for 30 minutes and DNA was visualized with a UV lamp.

III.B.6. Sample Preparation and Spreading for Electron Microscopy

A modification of the Wu and Davidson (1975) spreading technique was used. The binding of SSB to replicative products

was carried out in a mixture containing 0.01 M KPi at pH 7.4, 0.001 M Na_2EDTA, 130 μg/ml E. coli SSB and samples of reaction products at a final concentration of 1.94 μg/ml. After 10 minutes of incubation at 37°C, glutaraldehyde was added to a final concentration of 0.08%. The solution was incubated again for 15 minutes at 37°C and diluted 4-fold into a hyperphase solution containing 50 μg/ml ethidium bromide, 0.1 M Tris-Cl at pH 8.5, 0.001 M Na_2EDTA, 50 μg/ml cytochrome C and 0.4 M NH_4OAC. After spreading into a hypophase of 0.25 M NH_4OAC, samples were picked up on 3% Parlodion coated 200 mesh copper grids, stained with 0.5 μM uranyl acetate in 90% ethanol for 25 seconds, rinsed and dried in isopentane. After staining, the grids were rotary shadowed with Pt/Pd at an angle of 7°, and examined in a Zeiss EM10 Electron Microscope. Micrographs were taken on Kodak film. Positive prints were prepared on Agfa-Gevart Rapitone PF1-4.

III.B.7. Length measurement of single and double stranded DNA.

Double stranded regions of individual M13 DNA molecule (> 150 molecules per reaction product) were first marked using a 10x magnifying lens and the lengths of double strand were measured with a map measure. The value for % of total double stranded length of M13 DNA was obtained by dividing the length of double strand in each M13 DNA molecule by the length of RF M13 molecule spread

under the same condition (mean values of 50 RF molecules measured). For control experiments, both single and double stranded regions were measured and compared with the lengths of single stranded and RF M13 DNA molecules respectively. The single stranded M13 DNA control was spread in the presence of SSB and RF M13 DNA was spread in the presence of ethidium bromide.

III.B.8. Endonuclease Hae III restriction digest of replicative intermediates from the replication of singly primed M13 DNA by DNA polymerase α:primase complex and HSV-1 DNA polymerase.

Replicative intermediates were prepared as described above except 0.1 mM [α^{32}P]-dCTP (1337 cpm/pmol) was used as labeled substrate. Aliquots of 50 µl were removed at 10, 20, 40, 60 and 120 minutes. Reactions were terminated with the addition of EDTA and dCTP to final concentrations of 25 mM and 1 mM, respectively. To determine the extent of DNA synthesis, 5 µl aliquots were removed from each time point and pipetted onto glass fiber filters. The radioactivity was determined as described previously. The residual reaction mixtures were supplemented with carrier RF M13 DNA (25 µg/ml). The replicative intermediates were purified by extraction once with phenol and twice with chloroform:isoamyl alcohol (IAA). Briefly, equal volumes of phenol saturated with TE buffer were added to the DNA samples, mixed, centrifuged, removed and the bottom, organic layers discarded.

Equal volumes of chloroform:IAA (24:1) were added to the aqueous layers, mixed, centrifuged, removed and the bottom, organic layers discarded. The extraction procedure was repeated with equal volumes of chloroform:IAA. After centrifugation, the upper aqueous phases were transferred to a fresh tube, and the remaining organic layers were re-extracted with 25 µl TE buffer. Aqueous phases removed from the chloroform layer were supplemented with sodium acetate to a final concentration of 0.3 M. DNA was precipitated with ethanol, centrifuged, washed with ethanol, dried and resuspended in 100 µl of TE buffer. To remove excess unincorporated [α^{32}P]-dCTP, the replicative intermediates were precipitated with ethanol, centrifuged, dried and dissolved in 50 µl of 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 100 µg/ml BSA and 1 mM DTT. The replicative intermediates were digested with 10 units of endonuclease Hae III and incubation was carried out at 37°C for 120 minutes. The reactions were terminated with the addition of EDTA and SDS to a final concentration of 25 mM and 1%, respectively. The reactions were then heated to 65°C for 15 minutes. After mixing with 6 µl of 10X loading buffer to 60 µl of sample, 15 µl of samples were loaded onto the 5% polyacrylamide slab gel (13 x 13 x 0.1 cm). The gel was electrophoresed in buffer B at 30 V for 18 hours and stained with ethidium bromide. The polyacylamide gel was then autoradiographed on Kodak X-Omat AR-5 film with an intensifying screen at -70°C.

IV. Results and Discussion

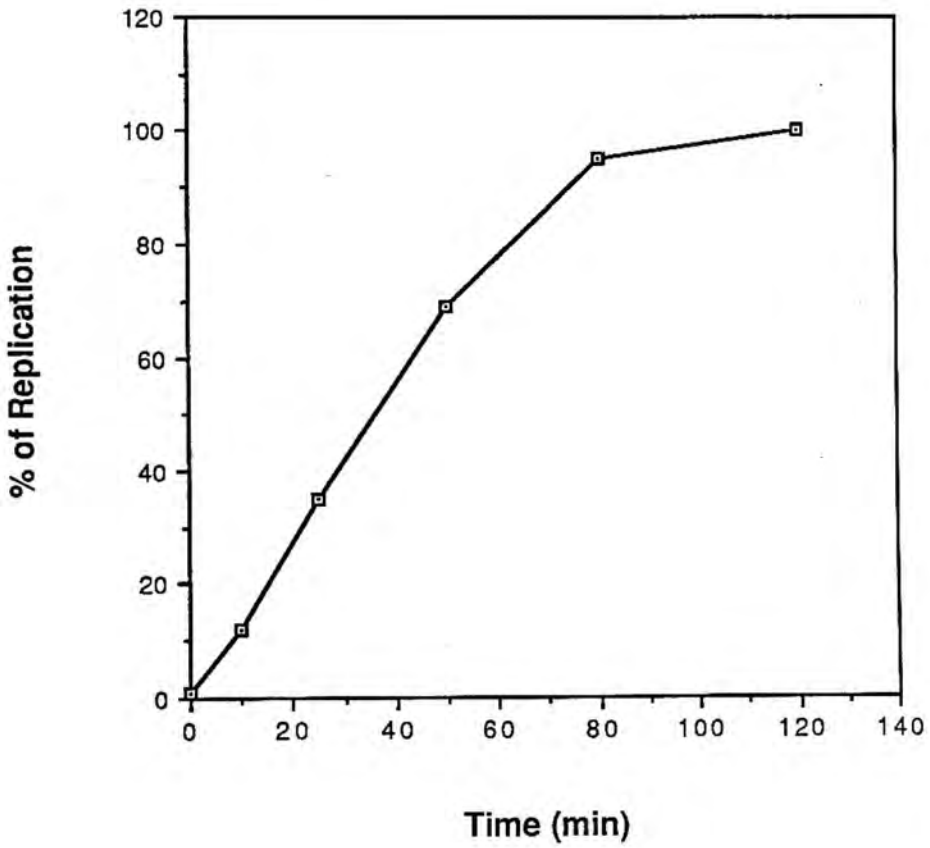
IV.A. Analysis of reaction products of replication of singly primed M13 DNA by calf thymus DNA polymerase α:primase complex.

The DNA polymerase α:primase complex has been shown by several authors to replicate a variety of templates including activated DNA, heat denatured DNA, ØX174 DNA primed with restriction fragment, oligonucleotide primed M13 and unprimed single stranded M13 DNA (Chang et al., 1984; Nasheuer and Grosse, 1987). Using the immunoaffinity purified DNA polymerase α:primase complex, the replication of singly primed M13 mp9 single stranded DNA was completed within 80 minutes as shown in Figure 2. The replication of single stranded M13 mp9 DNA by DNA polymerase α used a specific pentadecamer primer which base pairs to position 6667-6681 of the plus strand sequence. At an excess of primers over template, DNA polymerase copies M13 DNA very efficiently. Using the specific activity of DNA polymerase estimated with activated DNA (100,000 units per mg of protein), the number of enzyme molecules per pentadecamer primed M13 DNA in the reaction is ~4.

When samples of the reaction mixtures were withdrawn at 15, 30, 60 and 120 minutes and subjected to neutral agarose gel electrophoresis, the result showed that the template was

Figure 2. Time course of replication of singly primed M13 single stranded DNA by calf thymus DNA polymerase α:primase complex.

Singly primed M13 mp9 single stranded DNA (2.86 nM) was replicated containing a total of 118 units of DNA polymerase α: primase complex in the presence of four dNTPs, but in the absence of rNTPs. At the time indicated, aliquots were withdrawn and acid precipitable radioactivity was determined as described under Experimental Procedure.

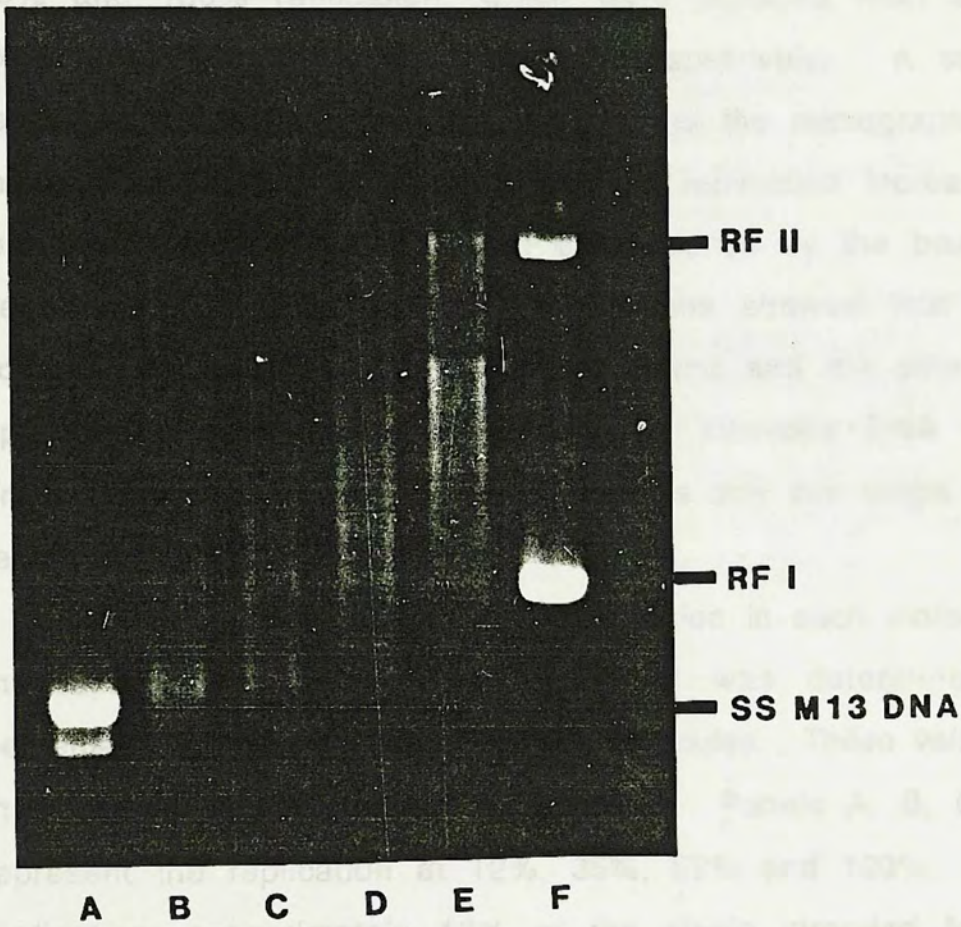

% of Replication
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (min)

eventually converted to a form that co-migrated with M13 RF (Figure 3). However, at early replication (15 minutes representing 15% replication) no full length products (RF II) were formed and instead the majority of DNA was in the region of the gel between primed single stranded DNA and RF II product.

In addition to examining the reaction products by gel electrophoresis, the RI were treated with E. coli SSB and mounted as described for electron microscopic analysis. There were several reasons why E. coli SSB was chosen over gene 32 protein for visualization of single stranded DNA. First was the SSB-single stranded DNA complex was easiler to visualize on the electron micrographs, because the diameter of the complex (10 nm) was thicker than that seen with gene 32 protein-single stranded DNA complex (8.5 nm). The second was that the SSB-single stranded DNA complex was more rigid and "jagged" which allowed a clearer distinction from the smooth double stranded DNA. The increased ability to distinguish the single stranded from the double stranded regions in the RI resulted in a more straight forward measurement of the product lengths. Furthermore, the apparent contour length of the single stranded M13 DNA in the presence of saturating amounts of SSB resulted in an approximately 35% reduction in contour length as compared to the RF II DNA. Whereas, the RF M13 DNA complexed with the ethidium bromide exhibited a smoother morphology with an increase of approximately 30% in the apparent

Figure 3. Neutral agarose gel electrophoresis of the products of singly primed M13 mp9 DNA replication by calf thymus DNA polymerase α:primase complex.

DNA products synthesized by DNA polymerase α: primase complex on singly primed M13 mp9 DNA (see Methods) were analyzed on 1% neutral agarose gel. Electrophoresis was for16 hours at 30 V. Following the staining with ethidium bromide, products were visualized with UV light. Lanes B-E represents replication intermediates at 15%, 26%, 51% and 94% replication respectively. Single stranded M13 mp9 DNA (lane A) and replicative form DNA (lane F) were used as size markers.


RF II
RF I
SS M13 DNA
A
B
C
D
E
F

DNA contour length as compared to RF II DNA spread in the absence of ethidium bromide. Figure 4 shows the RI present at 12%, 35%, 69% and 100% replication, which were obtained from a similar experiment (Panels A, B, C and D respectively). A schematic representation of each RI is adjacent to the micrographs. The micrographs of the RI showed that as replication increases, the length of single stranded region distinguished by the bound SSB decreases. In addition, the micrographs showed that SSB is continuously bound along the single strand and the difference in appearance between duplex and single stranded DNA in these preparation is clear. As expected, there is only one single stranded region in the DNA molecule.

The length of double stranded region in each molecule was measured, and the amount replicated was determined as a percentage of the total length of RF molecules. These values were then plotted in a histogram in Figure 5. Panels A, B, C and D represent the replication at 12%, 35%, 69% and 100%. At 12% replication, approximately 13% of the single stranded M13 DNA molecules appeared to be unchanged within the limit of detection and the rest appeared to be partially replicated. However, no RF II was seen at 12% or 35% replication. On the other hand, at later time (Panel D), some RF II could be seen but the majority of the reaction products remained partially replicated. At 69% replication, the reaction intermediate showed a much broader

Figure 4. Electron micrographs of replicative intermediates from the replication of singly primed M13 DNA by calf thymus DNA polymerase α: primase complex.

Reaction products as described in Figure 2 were treated with E. coli SSB and fixed with 0.08% glutaraldehyde. The samples were diluted in ethidium bromide buffer and spread with 0.4 M NH_4OAc and a hypophase of 0.2 M NH_4OAc. Panels A, B, C and D represent replicative intermediates at 12%, 35%, 69% and 100% replication, respectively. The micrograph is interpreted in the tracing adjacent to it.

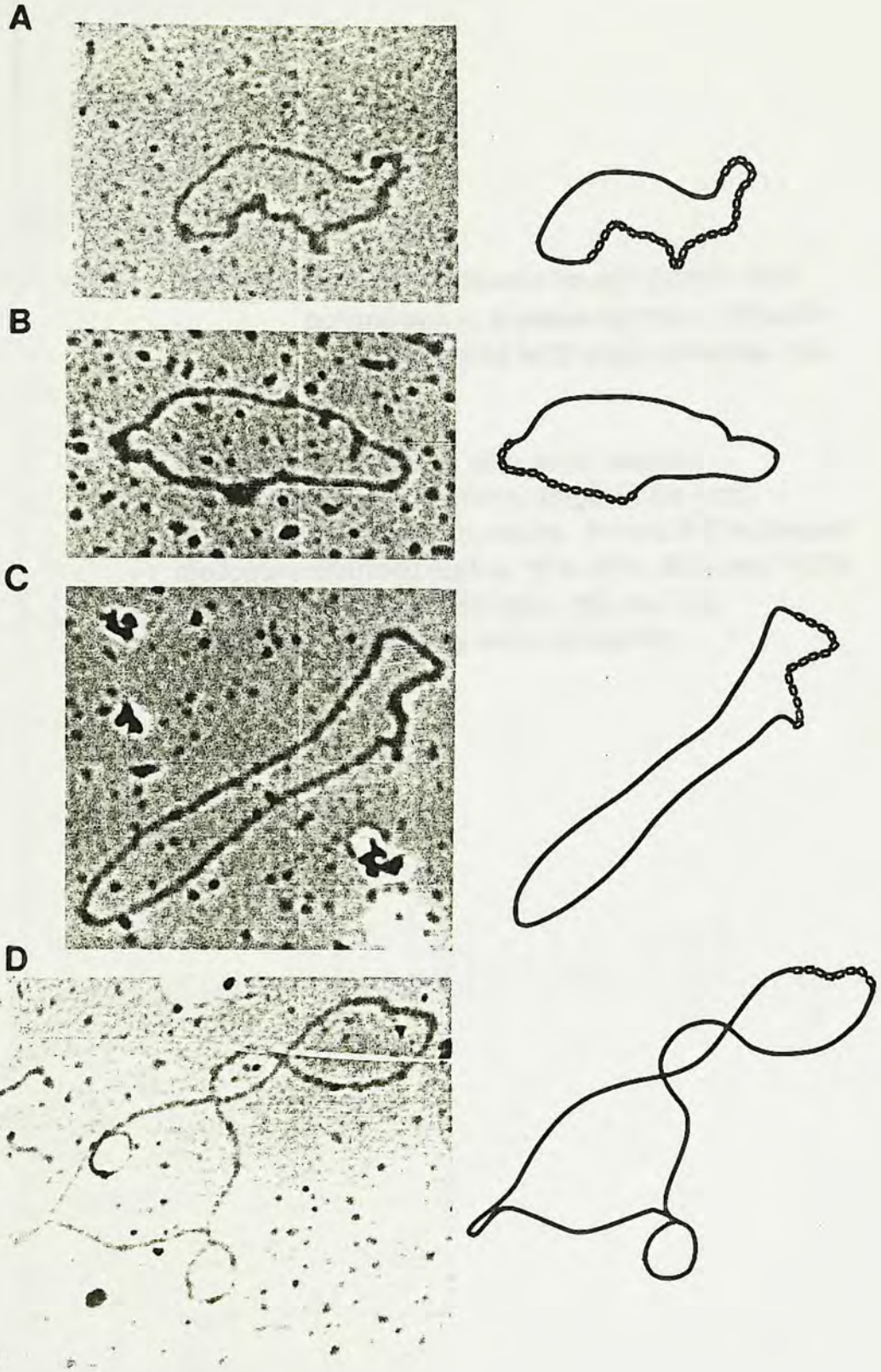
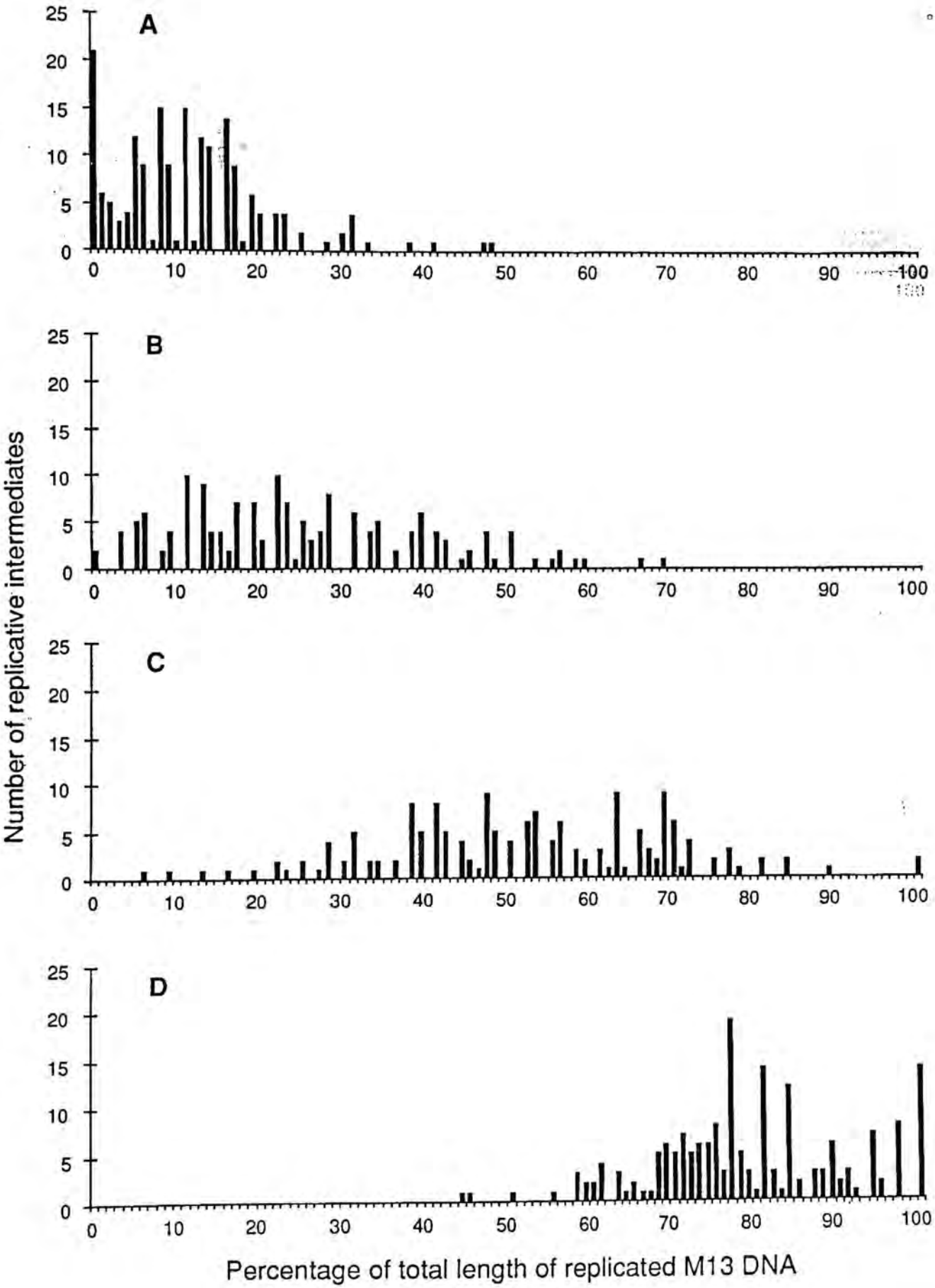

A
B
C
D

Figure 5. Product distribution for calf thymus DNA polymerase α: primase complex replication on singly primed M13 single stranded mp9 DNA.

Histogram showing the position of reaction intermediates as a fractional length of the total M13 double stranded molecule. Panels A-D represent replicative intermediates at 12%, 35%, 69% and 100% replication. A total of 166, 164, 162 and 186 molecules, respectively were measured.


A
B
C
D
Number of replicative intermediates
Percentage of total length of replicated M13 DNA
0
5
10
15
20
25
0
10
20
30
40
50
60
70
80
90
100

distribution which may suggest retardation of the DNA polymerase α during replication due to secondary structural barriers present on single stranded M13 DNA. Single stranded M13 DNA molecules have been shown to contain several secondary structures (Reckmann et al., 1985) that may serve as barrier to replication. Therefore, as DNA polymerase α replicates, these secondary structures may serve as a temporary barrier to replication but are eventually passed by the DNA polymerase α. The results shown in panel C indicate various sizes of intermediate product perhaps caused by temporary barrier to DNA polymerase, and some fully replicated products which indicated that the barriers had been passed by the DNA polymerase.

IV.B. Analysis of reaction products of replication of singly primed M13 DNA by herpes simplex virus type-1 DNA polymerase.

It has been reported that the HSV-1 DNA polymerase is stimulated 20-fold in the presence of E. coli SSB (O'Donnell et al, 1987). To examine the effect of E. coli SSB on the replication of singly primed M13 DNA, a time course assay was performed using purified HSV-1 DNA polymerase. The results shown in Figure 6 do not agree with the finding by O'Donnell et al, because under comparable conditions, the results showed little or no effect of SSB on the replication of singly primed M13 DNA. Although there was a

slight increase (10-20%) after 60 minutes, the initial rate of polymerization was essentially the same. A possible explanation for this may be due to differences in the preparation of E. coli SSB and HSV-1 DNA polymerase.

For comparison purposes, the replication assay was performed under condition similar to that of O'Donnell et al.. Synthesis of singly primed M13 DNA by HSV-1 DNA polymerase reached a plateau value at around 40-50 minutes which was determined to represent 65-70% replication of total single stranded M13 DNA (Figure 7).

When samples of the reaction mixtures were withdrawn at 10, 20, 30 and 120 minutes and subjected to neutral agarose gel electrophoresis, the result showed that some of the templates were eventually converted to a form that co-migrated with M13 RF DNA (Figure 8). Like the calf thymus DNA polymerase α:primase complex, no RF form was seen at the early time point, representing 7, 14 and 21% replication (Panels B-D), but at 65% replication, a portion of the single stranded M13 DNA had been converted to RF M13 DNA.

To determine the length of the products synthesized, samples prepared from a similar experiment as described in Figure 6 were purified, treated with E. coli SSB and mounted for electron microscopic analysis. Figure 9 shows the micrographs representing the replicative intermediates at 14%, 31% and 74% replication (Panels A, B and C respectively). Similar to the singly primed M13

Figure 6. Effect of E. coli single stranded binding protein on M13 DNA replication by herpes simplex virus type 1 DNA polymerase

Singly primed single stranded M13 mp9 DNA (2.86 nM) was incubated with 104 µg/ml of E. coli SSB for 15 minutes at 35°C. Replication was initiated by the addition of 4.4 units of HSV-1 DNA polymerase. Samples (20 µl) were removed at the time indicated, and acid precipitable radioactivity was determined as described under Experimental Procedures. -●-, No addition; -□- , single stranded DNA coated with E. coli SSB.

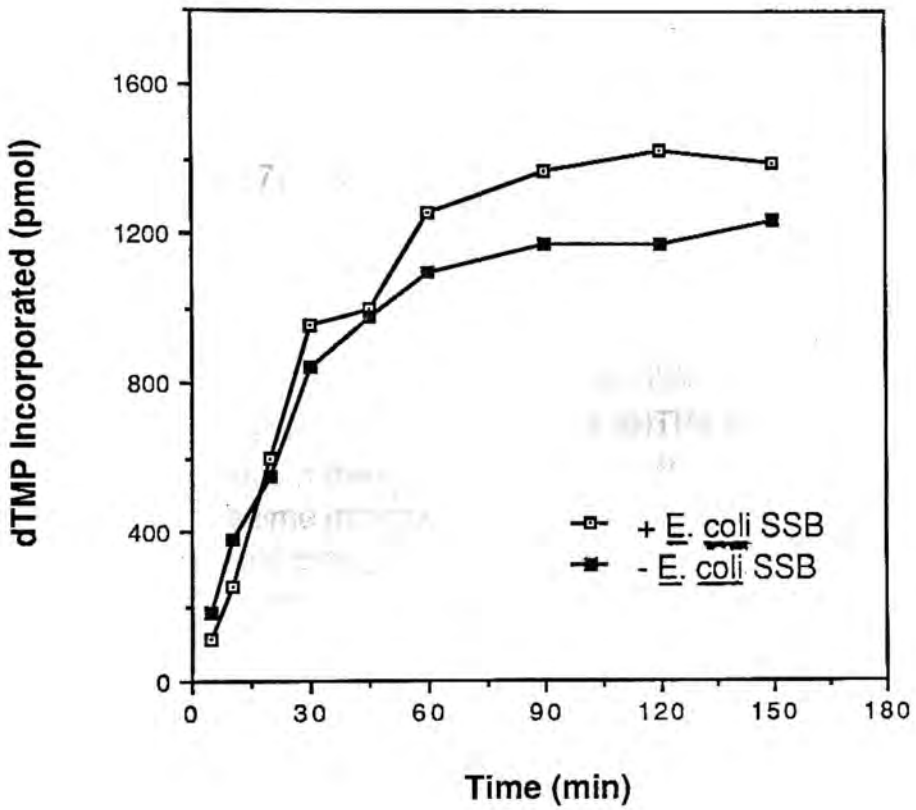

dTMP Incorporated (pmol)
1600
1200
800
400
0
0
30
60
90
120
150
180
Time (min)
+ E. coli SSB
- E. coli SSB

Figure 7. Time course of replication of singly primed M13 single stranded DNA by herpes simplex virus type 1 DNA polymerase.

Singly primed M13 DNA (2.86 nM) was replicated in the standard assay with 4.4 units of HSV-1 DNA polymerase in the presence of dNTPs and 115 μg/ml of SSB but in the absence of ribonucleoside triphosphates. At the time indicated, aliquots were withdrawn and acid precipitable radioactivity was determined as described in Experimental Procedures.

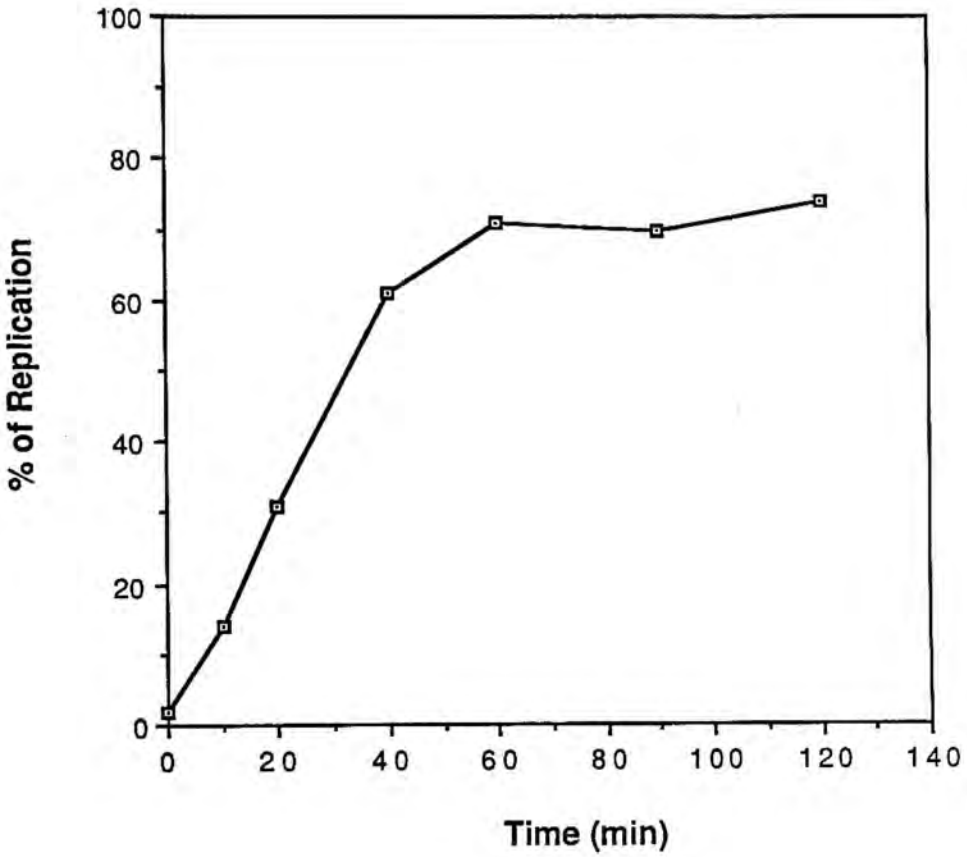
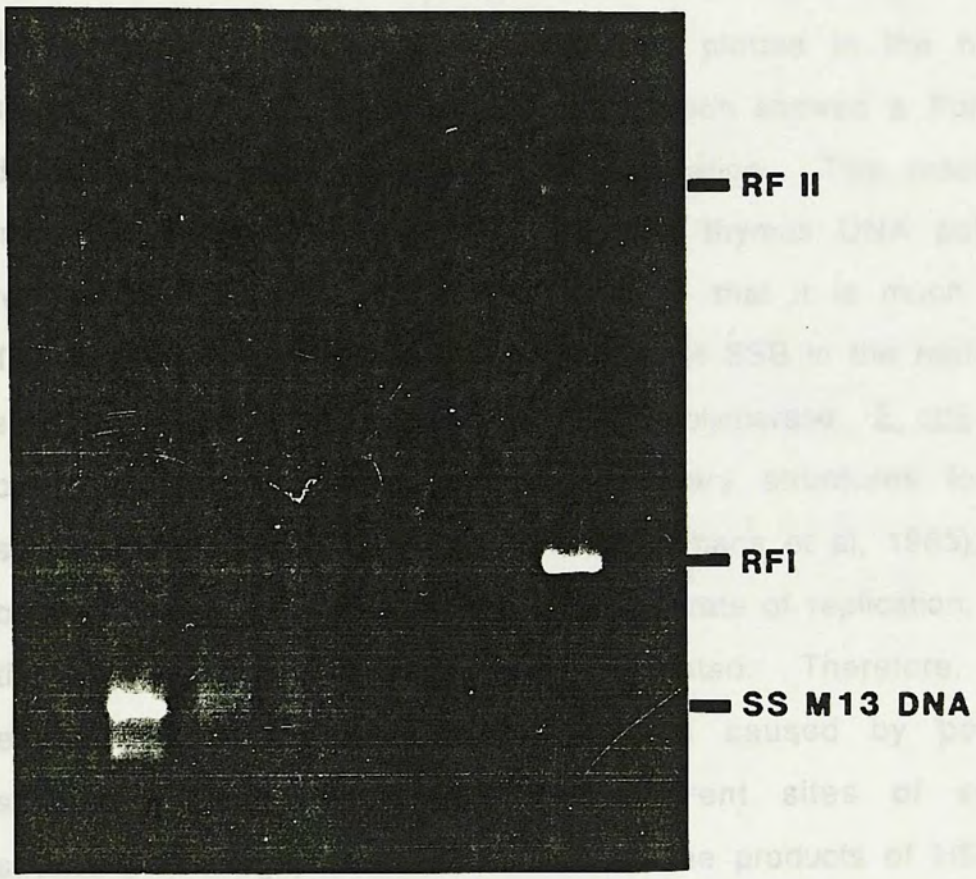

100
80
60
40
20
0
% of Replication
0
20
40
60
80
100
120
140
Time (min)

Figure 8. Neutral agarose gel electrophoresis of the products of singly primed M13 mp9 DNA replication by herpes simplex virus type 1 DNA polymerase.

DNA products synthesized by HSV-1 DNA polymerase on singly primed single stranded M13 DNA were analyzed on 1% neutral agarose gel. The gel was electrophoresed for 16 hours at 30 V. Following staining with ethidium bromide, products were visualized with UV light. Lanes B-D represents replicative intermediates at 7%, 14%, 21% and 65% replication respectively. Single stranded M13 mp9 DNA (Lane A) and RF M13 DNA (Lane F) were used as size markers.


RF II
RFI
SS M13 DNA
A
B
C
D
E
F

DNA replication by calf thymus DNA polymerase α:primase, the schematic representation of the RI showed that the increase in the length of double stranded DNA corresponded to the increase in replication time. By measuring the double stranded region of the RI the percentage of total RF length was plotted in the histogram shown in figure 10. The product distribution showed a Poisson-like distribution in both 14% and 31% replication. This result differs from the distribution seen with the calf thymus DNA polymerase replication of singly primed M13 DNA in that it is much sharper. The difference may be due the presence of SSB in the replication of single stranded M13 DNA by HSV-1 DNA polymerase. E. coli SSB has been shown to destabilize the secondary structures formed by single stranded M13 DNA molecule (Reckmann et al, 1985). In this case, while E. coli SSB did not alter the rate of replication, some of the pause sites may have been eliminated. Therefore, with the elimination of various product lengths caused by polymerase stuttering, and overcoming the different sites of secondary structure on single stranded M13 DNA, the products of HSV-1 DNA polymerase replication in the presence of SSB should be fairly uniform in length. Another possibility is that the HSV-1 DNA polymerase is simply more efficient in overcoming the secondary structural sites on the M13 DNA than calf thymus DNA polymerase α:primase complex.

Figure 9. Electron micrographs of replicative intermediates from the replication of singly primed M13 DNA by herpes simplex virus type 1 DNA polymerase.

Reaction products as described in Figure 7 were treated with E. coli SSB and fixed with 0.08% glutaraldehyde. The samples were diluted in an ethidium bromide containing buffer and spread with 0.4 M NH_4OAc and a hypophase of 0.25 M NH_4OAc. Panels A, B and C represent replicative intermediates at 14%, 31% and 74% replication.

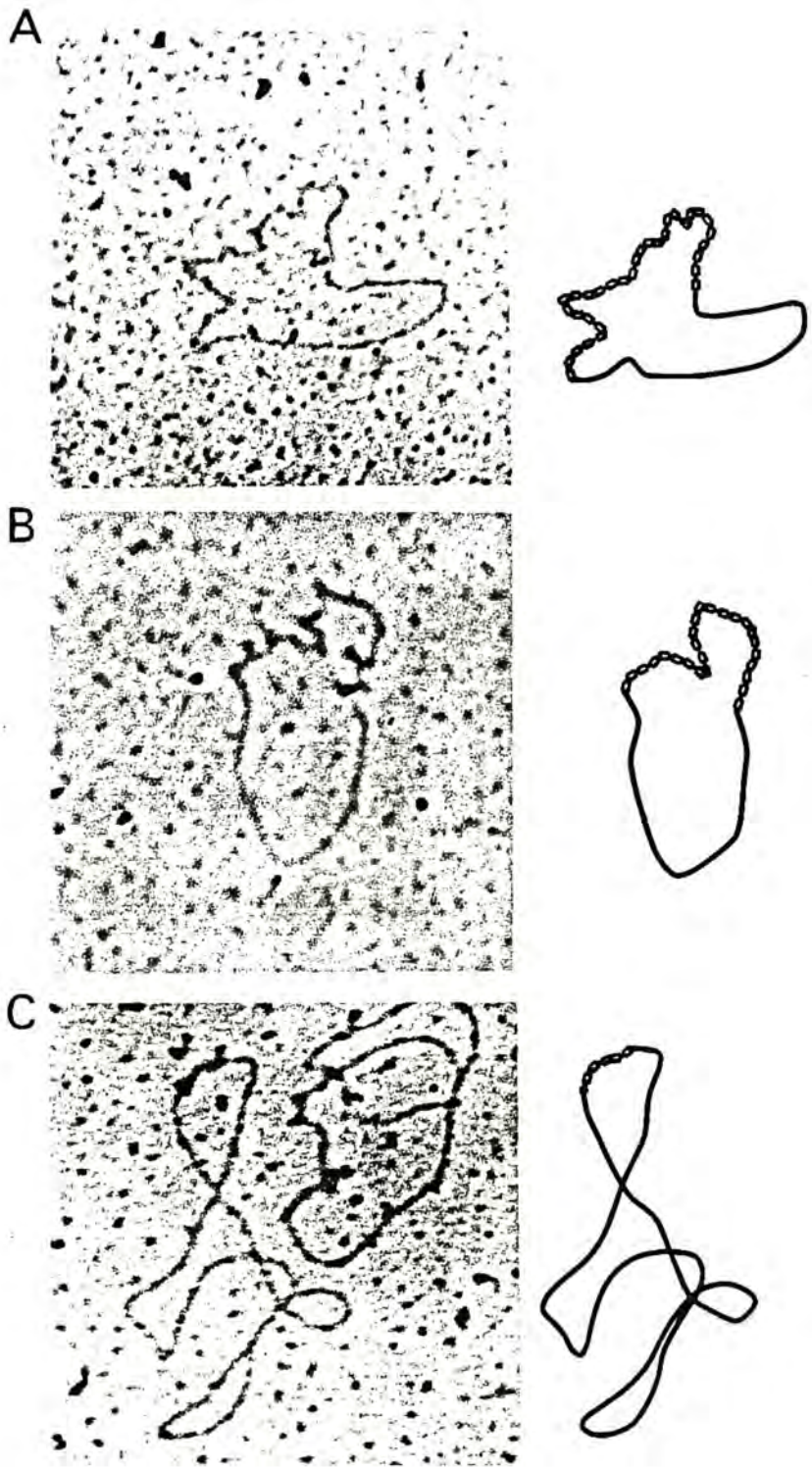
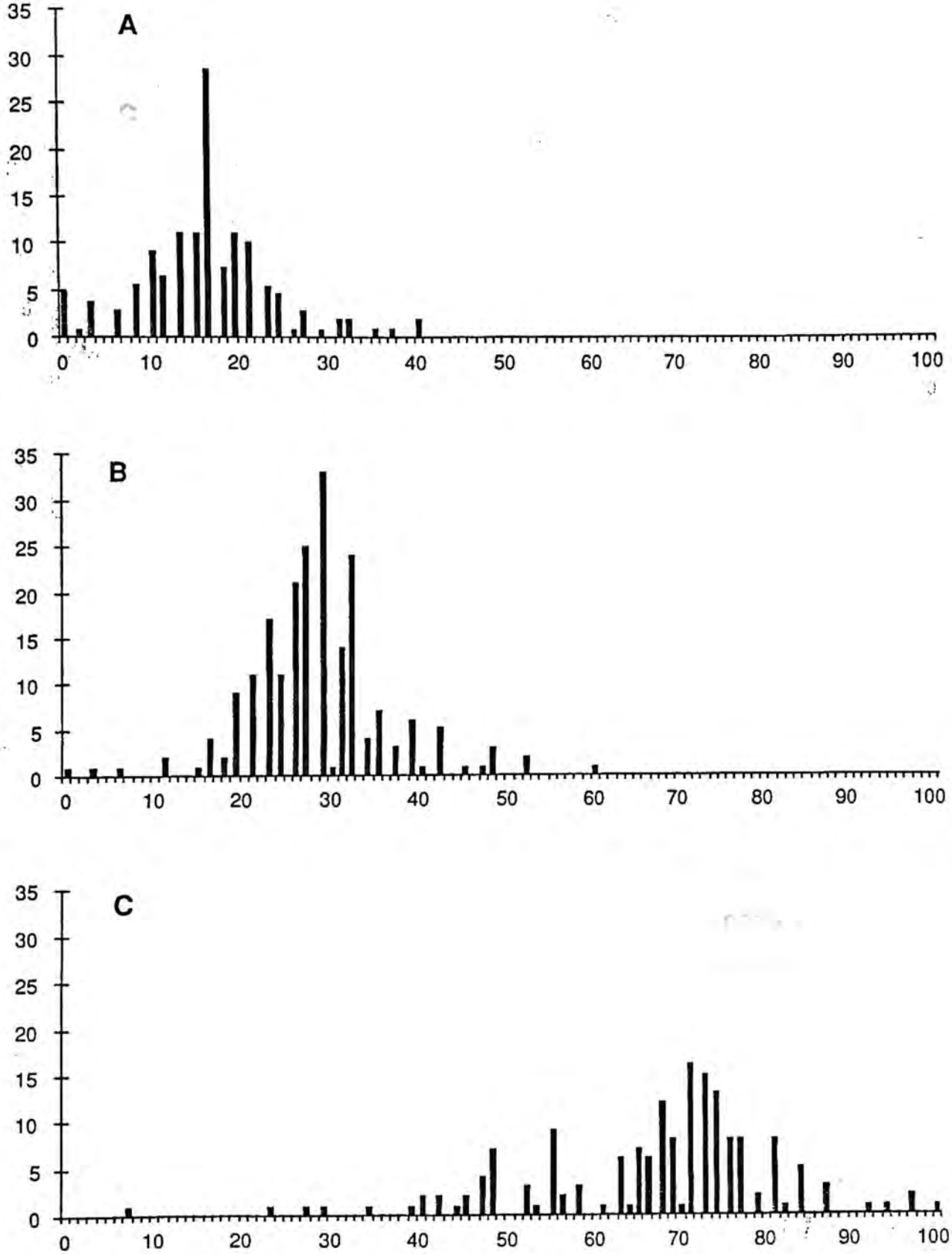

A
B
C

Figure 10. Product distribution for herpes simplex virus type 1 DNA polymerase replication of singly primed M13 single stranded mp9 DNA

Histograms showing the position of replicative intermediates as a fractional length of the total M13 double stranded molecule. Panels A-C represents replicative intermediates at 14%, 31% and 74% replication. A total of 150, 217 and 172 molecules, respectively were measured

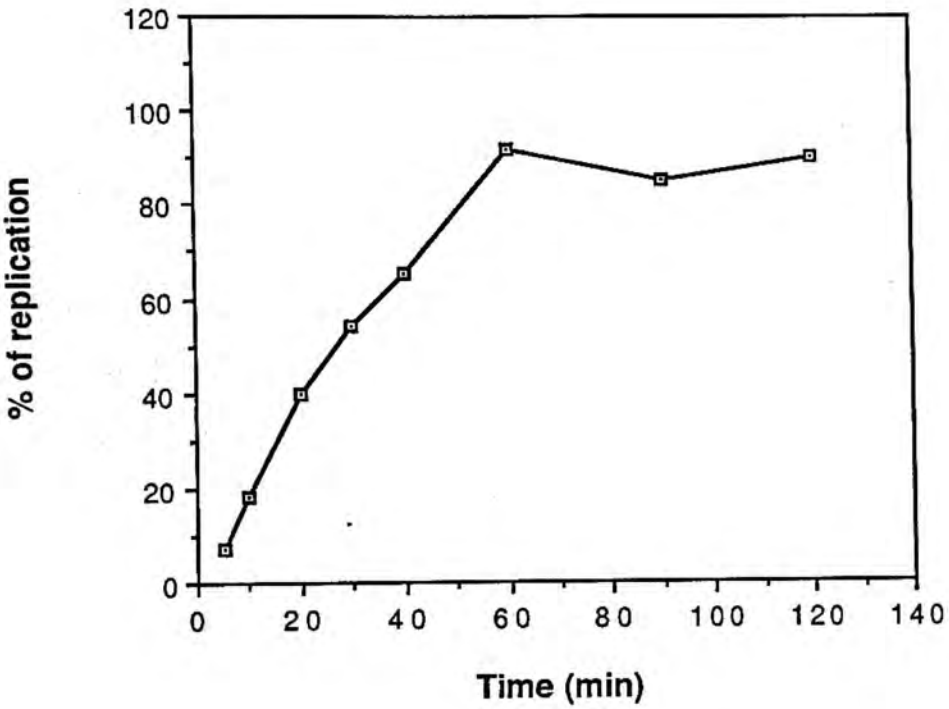

A
B
C
Number of replicative intermediates
Percentage of total length of replicated M13 DNA
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
70
80
90
100

IV.C. Analysis of reaction products of replication of primase primed single stranded M13 DNA by calf thymus DNA polymerase α:primase complex.

It was reported that replication of primase primed single stranded M13 DNA molecules in the presence of all four ribonucleoside triphosphates was much faster than replication of singly primed M13 DNA (Nasheuer and Grosse, 1987). Using a ratio of two molecules of enzyme per molecule of single stranded M13 DNA (2 fold increase of M13 DNA concentration as compared to singly primed reaction), M13 DNA synthesis levelled off at about 60 minutes (Figure 11), reaching greater than 90% of its theoretical value (ie, complete replication of M13 DNA). The extent of multiprimed DNA synthesis was similar to that of singly primed DNA. The more efficient replication seen in the primase primed M13 DNA reaction can be explained by two reasons. First, the DNA primase has been previously suggested to form several primers per M13 DNA molecules (Nasheuer and Grosse, 1987). Instead of one molecule of DNA polymerase replicating a singly primed site, the DNA primase generates several priming sites per template molecules. The complete replication of M13 DNA molecule is faster in the multiprimed reaction than the singly primed reaction. Second, because the DNA polymerase α is purified as a complex containing a primase activity, the DNA polymerase α could conceivably began replication immediately after the minimum

Figure 11. Time course of replication of primase primed M13 single stranded DNA by calf thymus DNA polymerase α:primase complex.

Single stranded M13 mp9 DNA (4.76 nM) was replicated with polymerase α:primase in a reaction mix containing a total of 118 units of the enzyme. The reaction was carried out in the presence of the four ribonucleoside triphosphates and the four dNTPs as described in Experimental Procedures. At the time indicated, samples were withdrawn and acid precipitable radioactivity was determined as described under Experimental Procedures.


% of replication
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (min)

amount of ribonucleotide has been added by the primase. Therefore, the time it takes for the polymerase to recognize the primer terminus is decreased.

When samples of the multiprimed reaction mixtures were withdrawn at 10, 30, 45 and 120 minutes and subjected to 1% neutral agarose gel electrophoresis (Figure 12), the results showed most of the single stranded M13 DNA template was eventually converted to M13 RF II and III (RF III was probably due to the nicked M13 DNA in this set of experiments). During the early replication (12% and 38%), virtually no full length product (RF II) was formed and instead the majority of DNA migrated near the position of primed single stranded DNA.

The RI were then purified, treated with E. coli SSB and mounted for electron microscopy as described in Experimental Procedure. The micrographs shown in Figure 13 show several single stranded regions that are not connected, suggesting several primers were formed by the primase. From the data collected by the measurement of double stranded regions of each replicative intermediate, it was found that at 18% replication, an average of 2.5 primers were formed per M13 genome. However, at 90% replication, only an average of one primer was detected. The discrepancy may be a result in the difficulty in detecting the unreplicated region between the two double strands where they had come close together. The sensitivity of using electron microscopy for detection of double stranded length is in the range of 150 to 200

Figure 12. Neutral agarose gel electrophoresis of primase primed M13 mp9 DNA replication by calf thymus DNA polymerase α:primase complex.

DNA products primed and synthesized by DNA polymerase α:primase complex on single stranded M13 mp9 DNA as described in Figure 10 were subjected to 1% neutral agarose gel and electrophoresed for 18 hours at 30 V. Products were visualized with UV light following ethidium bromide staining. Lanes B-E represent replicative intermediates at 12%, 38%, 54% and 98% replication respectively. Single stranded M13 mp9 DNA (Lane A) and RF M13 DNA (Lane F) served as a length standard.

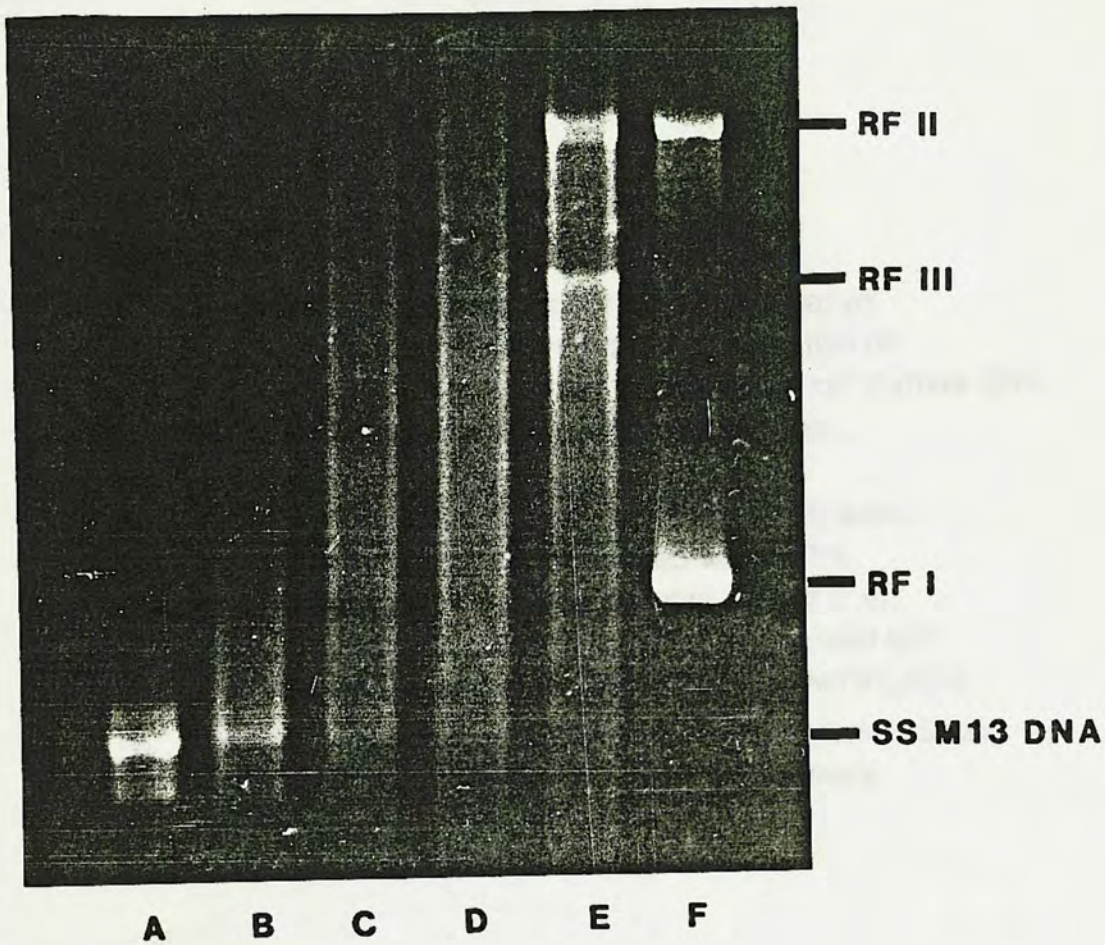
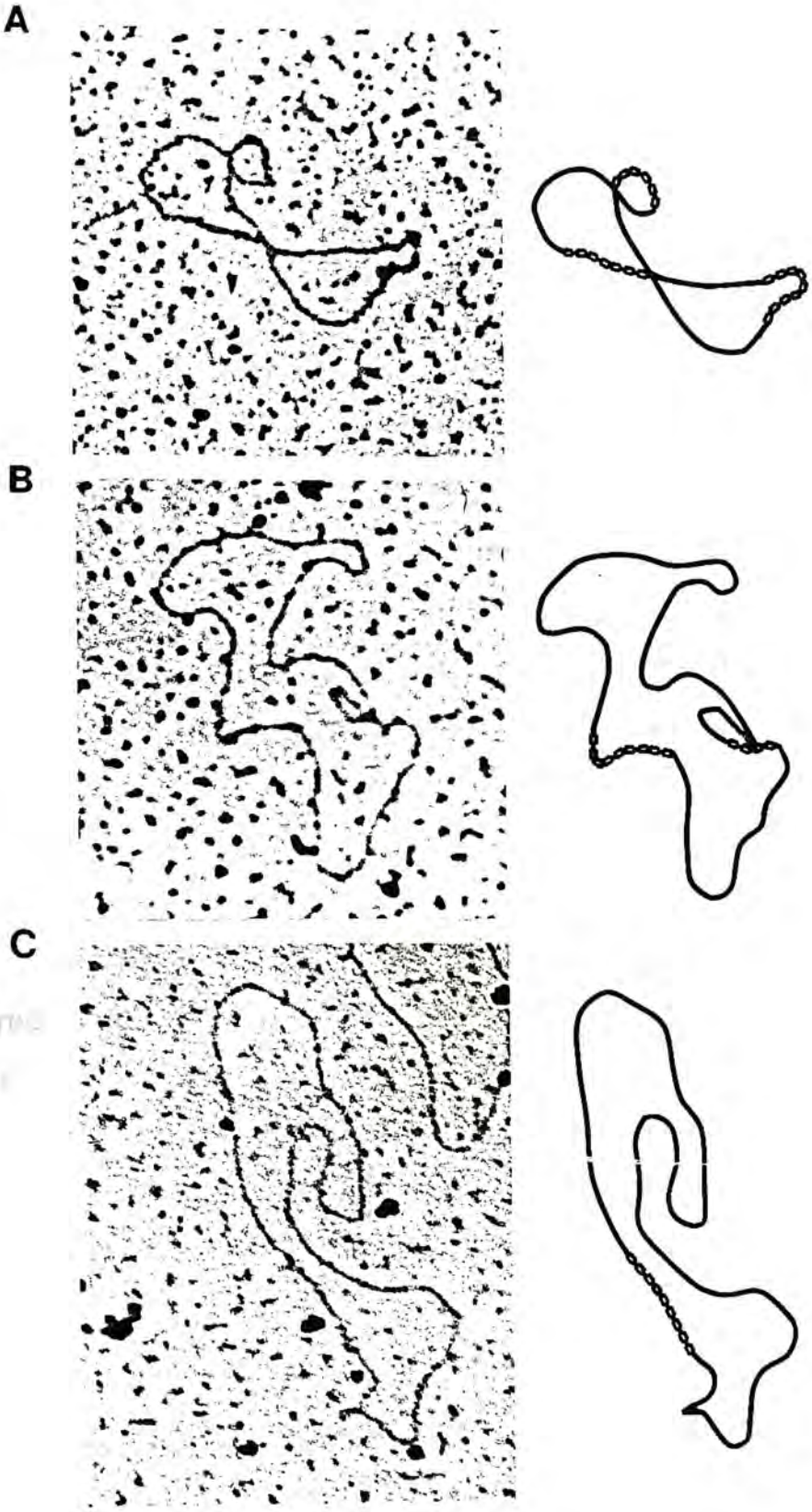

RF II
RF III
RF I
SS M13 DNA
A
B
C
D
E
F

Figure 13. Electron micrographs of replicative intermediates from the replication of primase primed M13 DNA by calf thymus DNA polymerase α:primase complex.

Reaction products as decribed in Figure 10 were treated with E. coli SSB and fixed with 0.08% glutaraldehyde. The samples were diluted in an ethidium bromide containing buffer and spread with 0.4 M NH_4OAc and a hypophase of 0.25 M NH_4OAc. Panels A, B and C represents replicative intermediates at 18%, 54% and 90% replication, respectively.


A
B
C

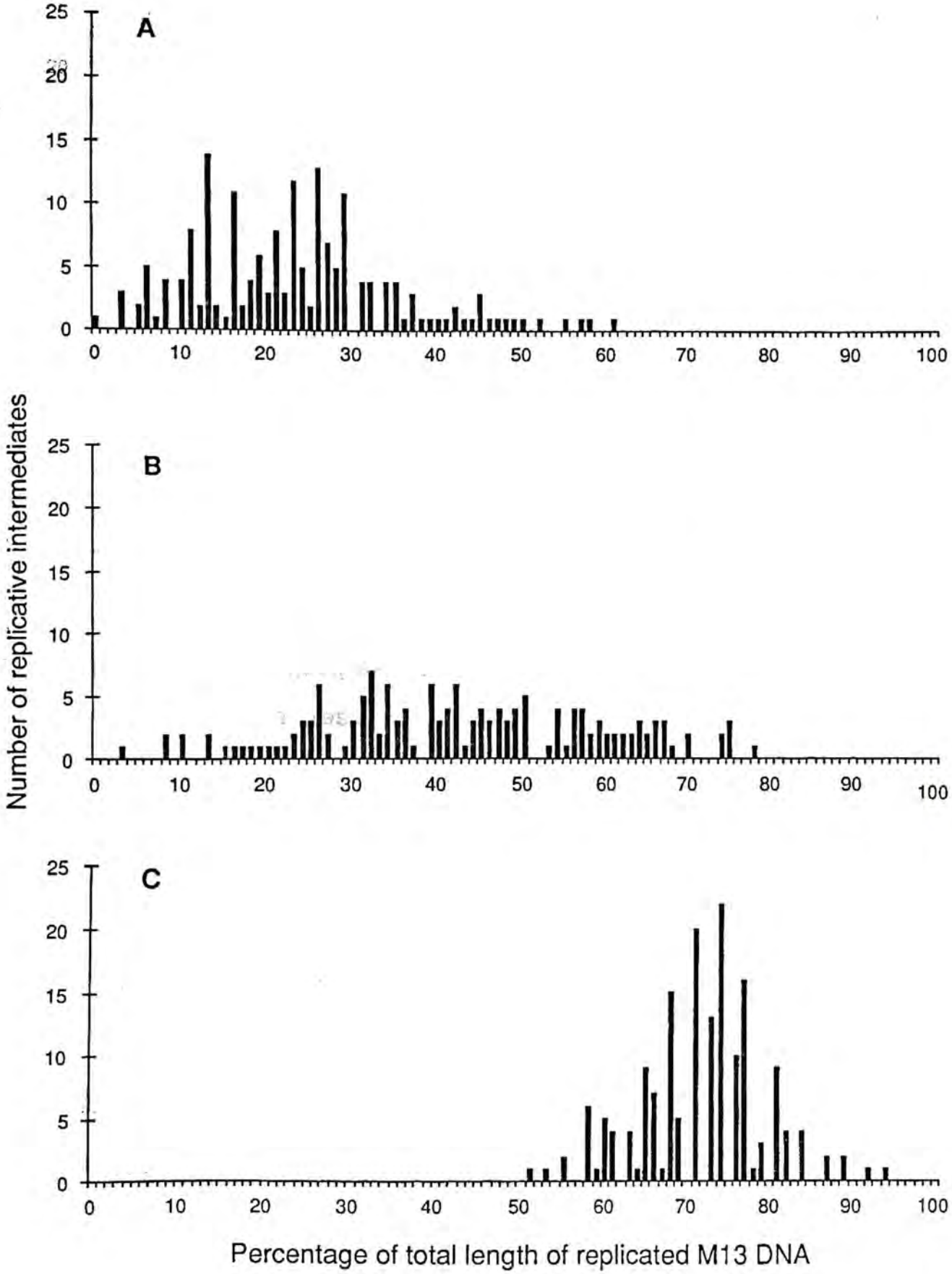
base pairs. Therefore, in a completely replicated template where the DNA template contains several gaps, the gaps were too small to be within the range of detection by electron microscopy.

The double stranded length of each RI was summed and represented as percentage of total length of RF molecule. These values were then plotted in a histogram shown in Figure 14. Similar to the singly primed M13 replication by calf thymus DNA polymerase α:primase complex, the primase primed M13 RI showed a broad distribution at 54% replication. In addition, like the previous two distributions, there are no fully replicated molecules formed at the early time point (18% replication), nor are there any totally unreplicated single stranded M13 molecules found at 90% replication.

When the double stranded length sections of each multiprimed RI were measured in the 18% replication, a distribution of product length was found from about 350 to 2,800 nucleotides. However, in the proceeding 54% replication, the average distribution of product length increased to about 2,200 to 4,500 nucleotides. The average number of primers for M13 single stranded DNA (2.5) were similar in both the 18% and 54% replication. This suggests that instead of more primers being synthesized by the primase, the initially synthesized primers were elongated by the DNA polymerase once a small number of primers were formed.

Figure 14. Product distribution for calf thymus DNA polymerase α:primase complex replication on primase primed M13 single stranded DNA.

Histogram showing the position of reaction intermedates as a fractional length of the total M13 double stranded molecules. Panels A-C represents replicative intermediates at 18%, 54% and 90% replication. A total of 184, 163 and 174 molecules were measured, respectively.


A
B
C
Number of replicative intermediates
Percentage of total length of replicated M13 DNA
0
5
10
15
20
25
0
10
20
30
40
50
60
70
80
90
100

In order to determine the accuracy of measurement at the boundary position between single stranded and double stranded region within the RI, the length of both single stranded and double stranded of each RI were measured. The values for single stranded and double stranded regions of RI were then divided by the length of respective single stranded M13 DNA and RF M13 DNA molecules to represent a percentage of unreplicated and replicated M13 DNA. If the measurement was totally accurate, then the sum of two percentages in a RI molecule would expected to be 100%. As seen in Table III, this is not alway the case. The mean values representing the sum of both percentage of single stranded and double stranded regions in RI were generally within the range of 100% even though the standard deviation was 10%. This significant standard deviation is a result of difficulties in detecting the specific interface between single stranded and double stranded DNA. The values obtained for the single and double stranded DNA are not a true representation of the product lengths. Twenty molecules from each % replication were selected based on the presence of both single and double stranded regions. Therefore, in the later % replication, only molecules containing significant length of single stranded DNA were selected to determine the accuracy of length measurement.

In all three product distribution profiles, the value of % replication is comparable to the product length measurement by electron microscopy in the early time points (12% to 50%).

Table III. Determination of the length of double stranded (DS) and single stranded (SS) regions of replicative intermediate.

I. Singly primed M13 DNA replicated by calf thymus DNA polymerase α:primase complex.

% Replication	SS DNA	DS DNA	SS DNA + DS DNA
12	79	15	94 ± 9
35	58	30	88 ± 10
69	45	47	92 ± 9
100	30	72	102 ± 11

II. Singly primed M13 DNA Replicated by HSV-1 DNA polymerase.

% Replication	SS DNA	DS DNA	SS DNA + DS DNA
14	82	16	98 ± 6
31	63	30	93 ± 8
74	34	66	100 ± 10

III. Multiprimed M13 DNA replicated by calf thymus DNA polymerase α:primase complex.

% Replication	SS DNA	DS DNA	SS DNA + DS DNA
18	74	27	101 ± 8
54	42	53	95 ± 8
90	29	77	106 ± 8

***Values for single stranded and double stranded measurement are expressed as the percentage of total length of respective single stranded M13 and replicative form M13 molecules. A total of 20 molecules were examined in each sample of % replication.

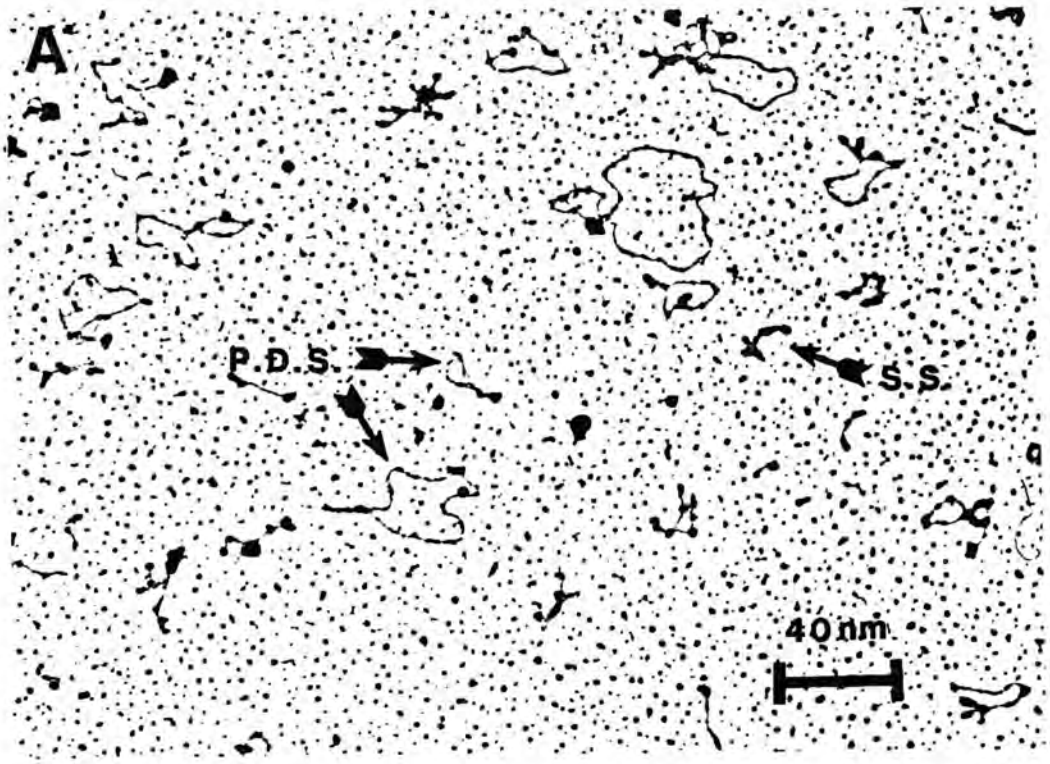
However, in the later replication, the product length measurements by electron microscopy were consistently below % replication, on the range of 10 to 25%. This difference in the measurement from % replication may be explained as follows. First, as noted previously, the error of measurement resulting from difficulties in detecting the region between single strand and double strand is approximately 200 to 400 nucleotides (3-5% standard deviation). This suggest that in DNA molecules where there are more interfaces between single strand and double strand, the error of measurement is likely to increase. Figure 14 showed that the average double stranded length is approximately 10-20% less than that of the calculated % replication and no completely replicated molecules were seen. Second, it is possible that the SSB used in the spreading condition may cause a small disruption in the double stranded DNA (region of high A-T) within the duplex as well as in the interface region of double stranded DNA. Therefore, the double stranded length may be underestimated due to the possible binding of SSB. Furthermore, if SSB is bound to a stretch of high A-T rich region within the duplex, the SSB-single stranded DNA complexes likely are not seen due to the limit of detection (150-200 nucleotides), but the measurements for double stranded length are reduced due to the conformations imposed on single stranded DNA by the SSB. This reduction of double stranded length would be more pronounced if there is more than one region where SSB binds within the duplex. This may explain why measurement of double stranded length in the later

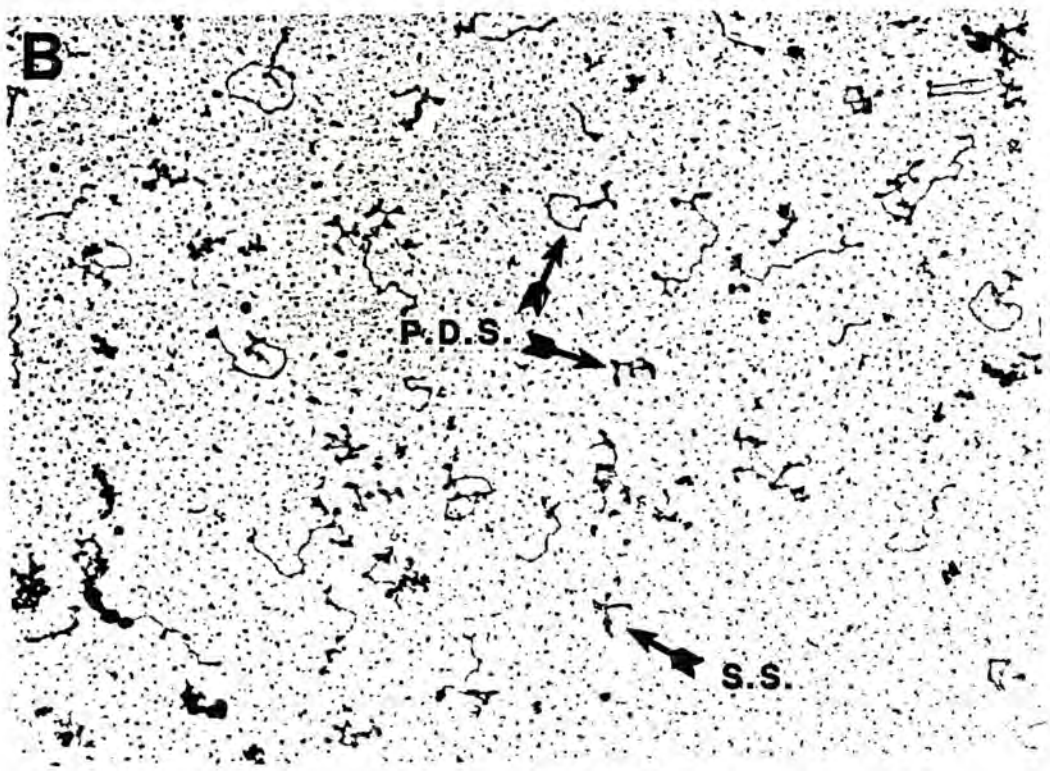
replication is less accurate than in early replication.

To achieve complete replication in a reasonable time, the ratio of DNA polymerase to DNA was adjusted so that fully replicated molecules could be achieved within 120 minutes and subsequently observed under electron microscopy. Because one cannot tell if the DNA polymerase is processive with excess DNA polymerase to M13 DNA, the reaction conditions were changed so that the molar concentration of M13 DNA was 2 to 3 fold greater than that of the DNA polymerase. With 3 fold excess of singly primed M13 DNA to calf thymus DNA polymerase α:primase complex, DNA synthesis was linear up to 4 hours. Reaction products were removed at 3 hours and purified as described in experimental procedures. The reaction products purified represented 28% replication. Using a 2 fold excess of singly primed M13 DNA to HSV-1 DNA polymerase, the reaction products were removed at 3 hours and were determined to represent 12% replication. The reaction products from both reactions were purified and mounted for electron microscopy in the absence of E. coli SSB and ethidium bromide. Under these condition, duplex DNA is extended while single stranded DNA is collapsed. This very obvious difference allows for a quick assessment of the sizes of DNA molecules showing extensive replication. In both cases of DNA replication, no fully replicated M13 DNA was seen which supports the view that both DNA polymerases replicate in a distributive manner (Figure 15).

Figure 15. Electron micrographs of replicative intermediates from both calf thymus and herpes simplex virus type 1 DNA polymerases replication of excess singly primed M13 single stranded DNA.

Singly primed M13 DNA (8.26 nM) was replicated in the standard assay with 110 units/ml of calf thymus DNA polymerase α:primase complex and 15.5 nM of singly primed M13 DNA was replicated in the similar condition with 1 unit/ml of HSV-1 DNA polymerase. After 3 hours of incubation, replicative intermediates were removed from both reactions and purified as described in Experimental Procedures. The replicative intermediates were spread with 0.4 M NH_4OAc and a hypophase of 0.25 M NH_4OAc. Panels A and B represent the replicative intermediates isolated from replication by calf thymus and herpes simplex virus type 1 DNA polymerases, respectively. Single stranded (S.S.) and partial double stranded (P.D.S.) DNA regions are identified.


A
P.D.S.
S.S.
40nm


B
P.D.S.
S.S.

Scoring all the molecules seen in the micrographs as either unreplicated, partially replicated or fully replicated, it was found that 36% of single stranded M13 remained unreplicated in the calf thymus DNA polymerase replication of M13 DNA, whereas 49% of single stranded M13 DNA remained unreplicated in the HSV-1 DNA polymerase replication (Table IV). These values may be overestimated due to difficulties in detecting small portions of double strand under the spreading condition. Also, it is possible that the DNA polymerases have higher affinity for partially replicated molecules than unreplicated ones. Third, because the reaction used in the assay to isolate the RI was carried out for 3 hours, this may result in the dissociation of the primer from the primer-template complex due to instability in the prolonged reaction. Thus, in the absence of primer to initiate DNA synthesis, the DNA polymerases are unable to replicate the single stranded M13 DNA.

Overall, the results obtained from DNA replication by calf thymus DNA polymerase α:primase complex are consistent with the previous observation that the DNA polymerase α replicates in a distributive manner during DNA synthesis. However, the distributive mode of DNA synthesis of HSV-1 DNA polymerase differs from the observation made by O'Donnell et al.. This difference in mechanism was not necessarily surprising since results obtained from the effects of SSB on replication of singly

Table IV. Distribution of replicative intermediates in both calf thymus and herpes simplex virus DNA polymerase replication of excess singly primed M13 single stranded DNA.

	Replication By:	
	Calf Thymus DNA Polymerase α:Primase Complex	HSV-1 DNA Polymerase
Number of molecule scored	258	190
Percentage of RI that are:		
A.) Single Stranded	36[a]	49[a]
B.) Partial Double Stranded	64	51
C.) Double Stranded	0	0

Replicative intermediates obtained from DNA synthesis by calf thymus DNA polymerase α:primase complex representing 28% replication and 12% replication from HSV-1 DNA polymerase DNA synthesis.

[a]Values may be overestimated due to inability to detect short double stranded regions.

primed M13 DNA by HSV-1 DNA polymerase also differed from the results from O'Donnell et al.. As mentioned previously, the differences may be a result of many factors; therefore, physical characterization of the HSV-1 DNA polymerase must be carried out beforehand to assure the homogeneity of the DNA polymerase, and then additional experiments for determination of processivity are needed to support the result suggested above. Electron microscopic analysis using SSB and ethidium bromide is necessary to examine the product distribution profile.

IV. D. Analysis of the reaction intermediates after restriction endonuclease Hae III digestion.

The reaction products from the replication of singly primed single stranded M13 DNA by calf thymus DNA polymerase α:primase complex and HSV-1 DNA polymerase were also examined by digestion with restriction endonuclease Hae III. According to the Hae III restriction map of RF M13 DNA (Figure 16), the appearance of restriction fragments primed at position 6667-6681 should be in the following order: Hae III-H (236), -J (173), -G (266), -I (214), -E (311), -N (106), -L (158), -A (2527), -F (309), -C (849), -B (1623), -D (341), -K (169), -M (117) and -O (102). Aliquots containing [α^{32}P]-labeled RI were removed at 10, 20, 40, 60 and 120 minutes from both DNA polymerase α and HSV-1 DNA polymerase reactions. The RI were purified with phenol/chloroform followed by

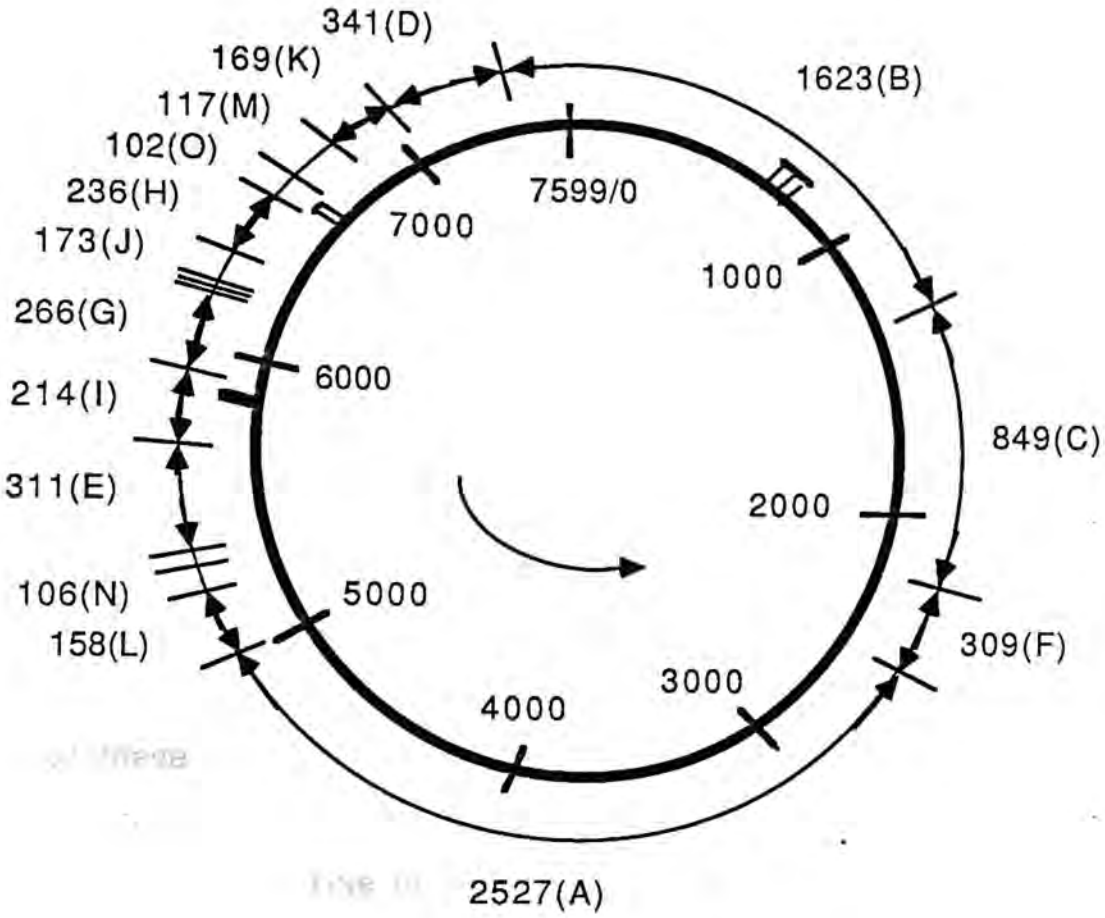


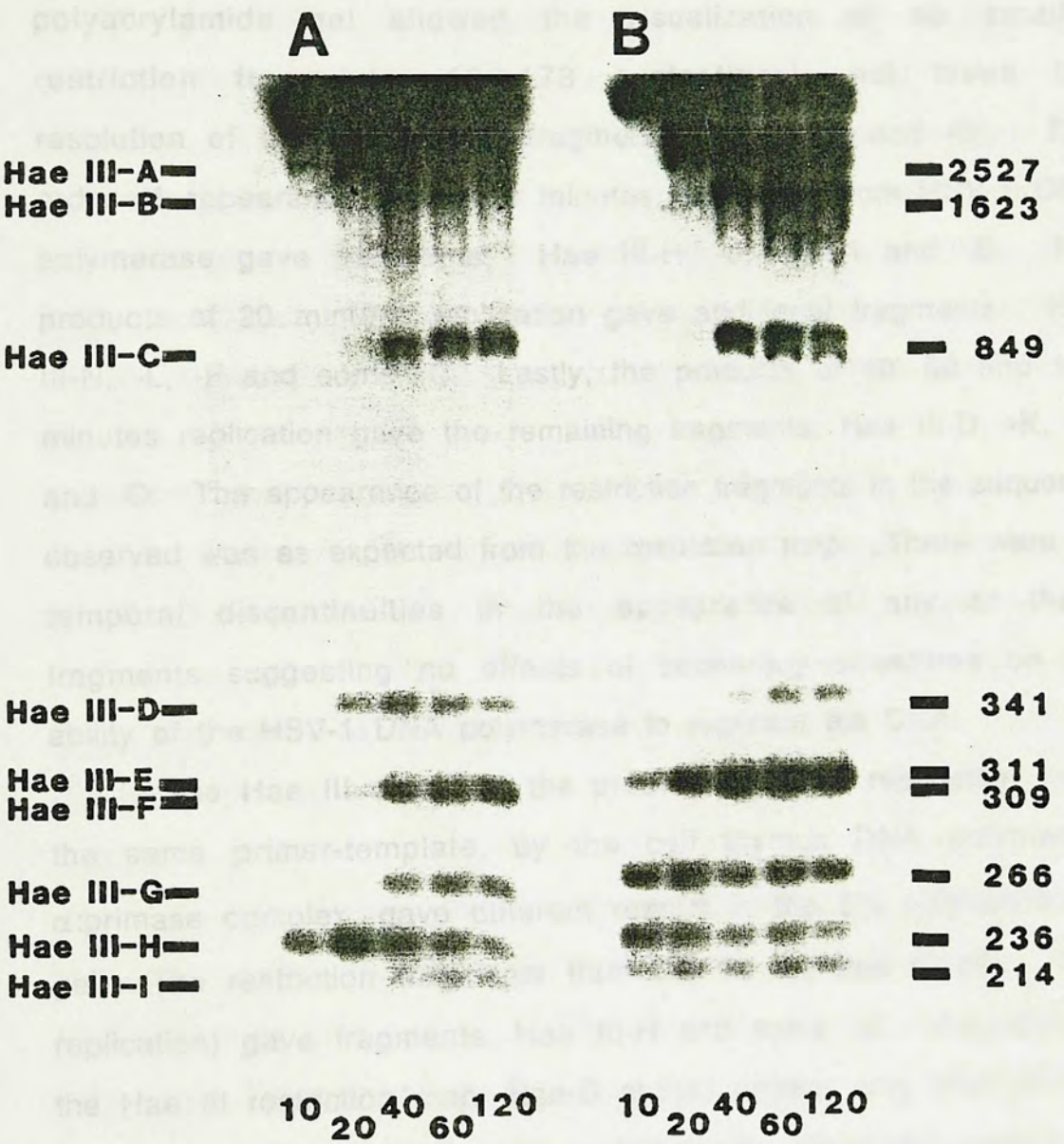
Figure 16. Restriction endonuclease Hae III map of RF M13 mp 9 DNA.

The arrow in the center represents the direction of replication. The specific hybridization site of the pentadecamer is located at position 6667-6681 (⊓). The major hairpin structures are located at position 5750 and 5792 (▮). The prominant aberrant event occurs within the region of 789-802 (⊓⊓). The numbers represented by the letters are the sizes of the restriction fragments.

precipitation with ethanol. The RI were then incubated with endonuclease Hae III, and restriction fragments were subjected to 5% polyacrylamide gel electrophoresis and visualized by autoradiography (Figure 17). The intensities of the band are proportional to the length of the restriction fragments. Under the electrophoresis conditions used, 10 restriction fragments (11 to 173 nucleotides) ran off the 5% polyacrylamide gel. In agreement with the expected order of appearance of restriction fragments, the digested 10 minutes replication products (representing 17% replication) from the HSV-1 DNA polymerase reaction, in the presence of SSB, gave fragments Hae III-H, -G, -I and -E. The sum of these fragments in terms of total nucleotides was 1215, which is approximately 17% of the total M13 genome. However, the absence of the Hae III-A band does not rule out that replication may have continued within this region. The products of the 20 minutes reaction (34% replication) gave fragments Hae III-A, -F (2 nucleotides less than Hae III-E) and some -C as well as the fragments of the 10 minute. Finally, the products of the 40 minute reaction (58% replication) and subsequent reaction products isolated at 60 and 120 minutes (69% and 72% replication, respectively), contained all the remaining restriction fragments, Hae III-B and -D. To examine the order of appearance of fragments smaller than the Hae III-I (214 nucleotide) fragment, the same samples of [α^{32}P]-labeled restriction fragments were subjected to

Figure 17. Restriction endonuclease Hae III analysis of replicative intermediates from singly primed M13 DNA replication by calf thymus DNA polymerase α:primase complex and HSV-1 DNA polymerase.

DNA products were synthesized in the presence of four dNTPs and [α^{32}P]-dCTP by calf thymus DNA polymerase α:primase complex and HSV-1 DNA polymerase on singly primed single stranded M13 DNA as described in Figures 2 and 7, respectively. At the time indicated, aliquots of [α^{32}P]-labeled replicative intermediates were removed and purified. The replicative intermediates were then digested with endonuclease Hae III and electrophoresed on a 5% polyacrylamide gel. The restriction fragments were visualized by autoradiography. Panels A and B showed the restriction fragments from M13 DNA replication by calf thymus DNA polymerase α:primase complex and HSV-1 DNA polymerase, respectively. The numbers at the right indicate the lengths (in nucleotide) of the restriction fragments.


A
B
Hae III-A
Hae III-B
Hae III-C
2527
1623
849
Hae III-D
Hae III-E
Hae III-F
Hae III-G
Hae III-H
Hae III-I
341
311
309
266
236
214
10 20 40 60 120
10 20 40 60 120

8% polyacrylamide gel electrophoresis (data not shown). The 8% polyacrylamide gel allowed the visualization of six smaller restriction fragments (102-173 nucleotides), but loses the resolution of the two largest fragments (Hae III-A and -B). The order of appearance in the 10 minutes replication from HSV-1 DNA polymerase gave fragments; Hae III-H, -J, -G, -I and -E. The products of 20 minutes replication gave additional fragments; Hae III-N, -L, -F and some -C. Lastly, the products of 40, 60 and 120 minutes replication gave the remaining fragments; Hae III-D, -K, -M and -O. The appearance of the restriction fragments in the sequence observed was as expected from the restriction map. There were no temporal discontinuities in the appearance of any of these fragments suggesting no effects of secondary structures on the ability of the HSV-1 DNA polymerase to replicate the DNA.

The Hae III digest of the products of DNA replication using the same primer-template, by the calf thymus DNA polymerase α:primase complex, gave different results in the 5% polyacrylamide gel. The restriction fragments from the 10 minutes reaction (12% replication) gave fragments, Hae III-H and some -D. According to the Hae III restriction map, Hae-D should appear only after 95% of singly primed M13 DNA has been replicated. When the products of the 20 minutes reaction (20% replication) were observed, the expected order of restriction fragments appeared; Hae III-H, -G, -I, -E, -A, -C and -D. The intensities of these bands were very weak,

while the intensities of Hae III-H and -D increased from the previous time point. This suggest that some singly primed M13 DNA are near complete replication while the majority remained near the region at 4% replication (Hae III-H). At 40 minutes reaction and thereafter, the band intensities of all the restriction fragments increased at the same rate suggesting that more M13 DNAs were being replicated. The appearance of extra restriction fragments in the 8% polyacrylamide gel supports the unusual results obtained in the 5% polyacrylamide gel. The products of the 10 minutes replication gave fragments; Hae III-H, -J and -D. The products of the 20 minutes replication contained fragments; Hae III-H, -J, -G, -E, -D, -K and some -C. The intensities of fragments Hae III-H, -J and -D increased while other fragments were relatively weak. Since the intensities of the bands are proportional to the length of the fragments as well as the number, then the absence of smaller fragments such as Hae III-N, -L or -I may be expected if these fragments are present in low concentration. In the 40 minutes replication, the majority of the bands were present except fragments Hae III-N and -O. The absence of these two fragments may again be a result of their low concentration and may reflect that these two fragments were under replicated. Finally, in the 60 and 120 minutes replication product digest, all the restriction fragments were present.

The interesting anomaly seen within the first 10 minutes

replication products of the calf thymus DNA polymerase α:primase complex may be due to an aberrant priming event. An aberrant priming by the pentadecamer primer has been seen previously in the replication of single stranded M13 mp7 DNA with conventionally purified calf thymus DNA polymerase α:primase complex (Grosse and Krauss, 1984). It was observed on a sequencing gel that at an excess of primer over template, an aberrant priming event occurred between position 789 and 803 of M13 DNA. When the primer concentration was greatly increased, further aberrant priming events occurred, but no priming site was located with certainty. This suggests that the hybridization of some primers at a template in this region had occurred between position 789 and 803, then the expected order of restriction fragments should first be Hae III-D followed by the order previously mentioned. Indeed, shown in both 10 and 20 minutes replication, restriction fragment Hae III-D appeared before all other fragments except Hae III-H and -J. In addition, fragment Hae III-K also appeared before fragment Hae III-I in the 20 minutes replication sample.

The appearance of Hae III-H and -J before other fragments is as expected since they are the first two fragments to be produced from a correct priming reaction. However, further replication of M13 DNA after the restriction fragment Hae III-J seems to be halted as seen with the delay in synthesis of subsequent fragments. This arrest of M13 DNA synthesis by DNA polymerase α is probably due to

a strong stable secondary structure. Secondary structures have been shown to delay DNA polymerase α during DNA synthesis (Weaver and DePamphilis, 1982). In the M13 genome, stable hairpin structures with the largest ΔG^{o} values are found in the origin of replication (Reckmann et al., 1985). The first structure is located at position 5750 and it serves as a signal for RNA polymerase to synthesize a short RNA primer (Gray et al., 1978). The second structure located at position 5792 serves as a signal for cleavage of supercoiled RF I by the endonuclease gene II-protein (Meyer and Geider, 1979). In the Hae III restriction map, these two hairpin structures are located within the region of fragment Hae III-I. In addition, analysis of recombinant DNA templates revealed that arrest sites are defined by sequences 140 bases downstream of the arrest point (Weaver and DePamphilis, 1982). This suggests that DNA synthesis by DNA polymerase α is halted within the region of Hae III-G. Therefore, the absence of Hae III-G and preceeding bands in the replication suggest that M13 DNA synthesis by calf thymus DNA polymerase α is strongly inhibited at these two hairpin structures. Furthermore, the appearance of Hae III-D before Hae III-G in the 10 minutes replication products suggests that DNA polymerase α probably elongates the primer at the aberrant priming site before overcoming the hairpin structures thus increasing the breadth of the distribution. At time during the replication reaction, the DNA polymerase α does overcome the hairpin structures as

shown with the appearance of the expected order of bands.

The results from M13 DNA replication by calf thymus DNA polymerase α:primase complex indicate that DNA polymerase α elongates predominately from the primer located at 6667-6681; however, the synthesis of M13 DNA is disrupted at the site of the origin of replication by stable hairpin structures. Instead of overcoming the secondary structures, the DNA polymerase α elongates primer aberrantly hybridized near bases 789 and 803 where no secondary structure is present. Elongation of the product primed from the primer eventually reaches the secondary structure upon which replication is temporary delayed. At the same time, some of the DNA polymerase α was able to overcome this major secondary structure and was not affected by other minor secondary structures present in the M13 genome as seen with the appearance of some of the restriction fragments in the 20 minutes replication. At the 40 minutes replication and thereafter, the majority of the DNA polymerase α molecules were able to overcome the barriers.

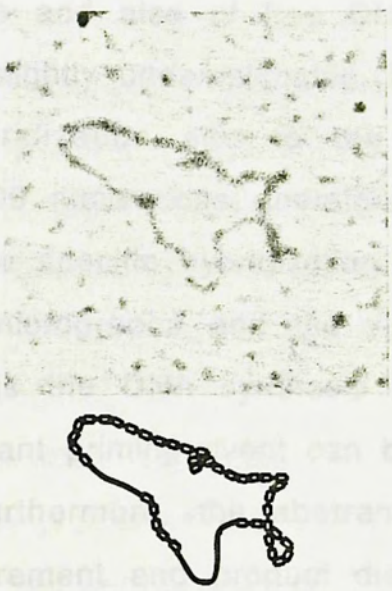
The results obtained from the replication by calf thymus DNA polymerase α:primase complex clearly differs from that of the HSV-1 DNA polymerase. As previously mentioned, progress of DNA synthesis by HSV-1 DNA polymerase does not seem to be affected by hairpin structures as shown with the sequentiai labeling of the expected order of restriction fragments. The absence of restriction fragment, Hae III-D in the 10 and 20 minute replication products

suggests that any hybridization of primer in this region is transient and once the primer dissociates from the template, SSB prevents its reassociation. Also, the presence of SSB may help the HSV-1 DNA polymerase to replicate through some secondary structures. Therefore, further experiments on HSV-1 DNA polymerase, in the absence of SSB, must be done to determine the effect of secondary structures on HSV-1 DNA polymerase replication.

Due to the aberrant priming event in the replication of singly primed M13 DNA by calf thymus DNA polymerase α:primase complex, it was of interest to see if the aberrant priming can be detected in the electron micrographs. Only the micrographs from early replication (12% and 35% replication) were examined because the distance from the most prominant aberrant priming site to the specific hybridization site is about 20% of the total M13 genome. Therefore, assuming that aberrant priming event is most likely to be observed during early replication due to the arrest of DNA polymerase α at the secondary structure site, replicative intermediates after 20%-25% replication will have one double stranded DNA region in the electron micrograph. Out of a total 164 molecules from the 12% replication, only 3% were determined as having two regions of double stranded DNA (Figure 18). On the other hand, electron micrographs from the 35% replication did not contain any unusual double stranded DNA. This suggests that at around 35%

Figure 18. Electron micrograph of a replicative intermediate illustrating an aberrant priming site.

Reaction product from 12% replication of singly primed M13 DNA by calf thymus DNA polymerase α:primase complex prepared for electron microscopy as described in Figure 4. The micrograph is interpreted in the tracing adjacent to it.



replication, most of DNA synthesis from the aberrant priming site probably had reached the specific priming site. Also, some of the delay of DNA synthesis caused by the hairpin structures may have been relieved. The relatively low value of aberrant priming event obtained in the 10% replication suggests that the aberrant priming event is probably not very common, but is possible if there is an excess of primers and also of free DNA polymerase. This low number may be slightly underestimated, because the distance from the specific hybridization site to the secondary structures is around 250 to 300 nucleotides; therefore, it is possible that DNA synthesis from the specific hybridization site may not be detected in the electron micrographs and the visualization of one double stranded region is the DNA synthesis from the aberrant primer. Overall, the aberrant priming event can be detected in the electron micrographs. Furthermore, the aberrant priming event does not affect the measurement and product distribution profiles, because of its low occurrence and because the appearance of the two double stranded regions per M13 DNA molecule is short-lived.

V. Conclusion

Using electron microscopic analysis, the results showed that both calf thymus DNA polymerase α:primase complex and HSV-1 DNA polymerase replicate singly primed M13 DNA in a distributive manner. However, to obtain complete replication and product length analysis by electron microscopy, excess DNA polymerase over template had to be used. The product length analysis by electron microscopy (spread with SSB and ethidium bromide) eliminated the problem of resolution seen in gel electrophoresis, because individual M13 DNA molecules are seen and the double stranded length can be measured. In addition, the number of primers synthesized by the calf thymus DNA primase can be determined from the number of double stranded regions seen on the primase primed M13 DNA. Results from the product distribution profiles and endonuclease Hae III restriction mapping showed that the calf thymus DNA polymerase α:primase complex did not replicate M13 DNA in a continuous manner but was delayed by stable hairpin structures. This result supports the finding that stable secondary structures of the single stranded DNA template can lead to a pausing of DNA polymerase α (Villani, et al., 1981; Reckmann et al., 1985; Weaver and DePamphilis, 1982). On the other hand, HSV-1 DNA polymerase, in the presence of SSB, does not seem to be affected by the hairpin structures. Although the electron microscopic analysis using SSB and ethidium bromide provided a good and quantitative method for determining the product

lengths, error in the measurement reduces its efficiency in obtaining accurate chain length. However, this error in measurement may be minimized by using a summagraphic digitizer rather than map measurer. The summagraphic digitizer traces the contour length of individual M13 DNA molecule to 0.1 mm resolution and the distance traveled is recorded into the computer. This technique allows a more accurate measurement especially at the bends of the DNA molecule. The map measurer is fairly accurate if the DNA molecule is linear, but the accuracy is reduced if the molecule is looped back causing difficulties in tracing the molecule precisely. Therefore, if the measurements were done using the summagraphic digitizer, then it is possible that some of the errors in measurement can be reduced.

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